AD_____

Award Number: W81XWH-09-1-0639

TITLE: Progesterone Receptor Scaffolding Function in Breast Cancer

PRINCIPAL INVESTIGATOR: Dr. Christy Hagan

CONTRACTING ORGANIZATION: The University of Minnesota
Minneapolis, MN 55455

REPORT DATE: October 2011

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-10-2011	Annual Summary	29 SEP 2010 - 28 SEP 2011

4. TITLE AND SUBTITLE
Progesterone Receptor Scaffolding Function in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0639

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dr. Christy Hagan

E-Mail: hagan018@umn.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Minnesota
Minneapolis, MN 55455

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Abstract on next page.

15. SUBJECT TERMS
Breast cancer, progesterone receptor

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	54	19b. TELEPHONE NUMBER *(include area code)*

14. ABSTRACT

Progesterone receptors (PR) are critical mediators of mammary gland development and contribute to breast cancer progression. Progestin-induced rapid activation of cytoplasmic protein kinases leads to selective regulation of growth-promoting genes by phospho-PR species. We have shown that phosphorylation of PR Ser81 is ck2-dependent, progestin-regulated and cell cycle-dependent in intact cells. Mutation of the CD domain in PR (mCD PR) abrogates phosphorylation on Ser81, indicating that the CD domain in necessary to facilitate phosphorylation at this site (Ser81). Additionally, we showed that an interaction between PR and MKP3, a regulator of the ERK family, is dependent on the CD domain. Regulation of selected genes by PR-B also required the CD domain for basal and/or progestin-regulated (STAT5A, Wnt1, MKP3) expression/repression. We conclude that the CD domain of PR facilitates protein interactions that are critical to PR-dependent transcription of genes involved in proliferation and mammary stem cell maintenance. Experiments to determine how MKP3 binding mechanistically regulates transcription of these genes, as well as phosphorylation at Ser81 (by ck2) are currently underway. Understanding how mitogenic protein kinases, such as ck2, alter PR phosphorylation and function is critical to fully understanding breast tumor etiology and developing better targeted therapies. Recent clinical data linking the progesterone component of hormone-replacement therapy regimens with the development of breast cancer underscores the importance of understanding how PR works in the context of breast cancer and high kinase environments. Due to the ubiquitous nature of ck2 and its prevalence in many different types of cancer, there has been much interest in the development of ck2 inhibitors as anti-cancer agents. Clinical ck2 inhibitors, in combination with more specific anti-progestins (new classes of selective progesterone receptor modulators or SPRMs), could provide an effective combination of targeted therapy for breast cancer treatment.

Table of Contents

INTRODUCTION

Progesterone receptors (PR) are critical for massive breast epithelial cell expansion during mammary gland development and contribute to breast cancer progression. Nuclear PR activates transcription of PR-target genes, either directly through binding to progesterone response elements (PREs), or indirectly through tethering interactions with other transcription factors (AP1, SP1, STATs). PR is highly post-translationally modified, primarily on N-terminal serine (phosphorylation) and lysine (ubiquitination and sumoylation) residues [1-3]. These modifications significantly alter receptor stability, localization, transcriptional activity and promoter selectivity [4]. In addition to MAPK and cdk2, casein kinase II (ck2), a kinase often overexpressed in breast cancer, has been shown *in vitro* to phosphorylate PR Ser81 [5-7]. Finally, recent clinical data has shown that women taking hormone-replacement therapy whose regimens included estrogen and progesterone, but not estrogen alone, had an increase in breast tumor number and size [8, 9]. In light of these data, understanding how mitogenic protein kinases alter PR is critical to understanding breast tumor etiology and developing better treatments. Progestin-bound PRs induce rapid activation of cytoplasmic protein kinases, leading to regulation of growth-promoting genes by transcription complexes that include phospho-PR species. We propose that hormonal and growth factor signals converge at the level of PR-target gene promoter selection. We identified a putative common docking (CD) domain in the N-terminal B-upstream segment (BUS) of PR-B. [10]. CD domains are regions through which MAPKs (i.e. ERK) interact with their activators, MAPK kinases (MKKs; i.e. MEK1) and inactivators, MAPK-phosphatases (MKPs) [10, 11]. Another nuclear receptor, PPARγ, has also been shown to interact with MEK1 through a similar domain [12]. The PR CD domain, DPSDE, is an exact match to the CD domain of ERK2, suggestive of PR direct binding with MEK1 and/or MKPs. We created a CD domain mutant (mCD PR) that is differentially post-translationally modified following treatment with synthetic progesterone (R5020), as indicated by its lack of phosphorylation-dependent gel retardation, or “up-shift”, when analyzed by Western blotting. These data suggest that mutation of the CD domain disrupts interactions with kinases that are responsible for direct phosphorylation of PR. Because mCD PR fails to up-shift upon ligand-binding, we screened for protein kinases whose target sequences are within close proximity of PR’s CD domain; PR Ser81 is a known ck2 site in the PR N-terminus. ck2 is a ubiquitously expressed, constitutively active kinase that is overexpressed in every cancer examined thus far, including breast cancer [5, 6]. Interestingly, in breast cancer cells treated with highly specific ck2 inhibitors, TBB and DMAT, we observed a loss of the progesterone-dependent PR up-shift, similar to the behavior of the mCD PR mutant. This affect on PR was specific to inhibition of ck2, as treatment with other kinase inhibitors did not affect PR gel mobility following treatment with R5020. These data suggest that ck2 may contribute to protein interactions and/or PR activity via direct phosphorylation of PR. Additionally, these data suggest that protein interactions mediated through the CD domain may affect PR Ser81 phosphorylation. We hypothesize that the PR CD domain mediates direct interactions with mitogenic protein kinases (MEKs, ck2) that phosphorylate PR, thereby dictating downstream signaling and target-gene specificity. In the context of breast cancer where protein kinases are inappropriately activated, hyperactive PR may lead to reprogramming of breast cancer cells, altering their hormone sensitivity and driving breast cancer progression.

BODY

Note: Highlighted text and figures indicate new additions to Annual Report.

MAJOR RESEARCH TASKS:

***Task 1*: Analysis of the signaling molecules that require the CD domain for PR docking (Months 1-12):**

Human PR exists in two primary isoforms: PR-B and PR-A. The full-length receptor, PR-B (116 kDa), contains a unique N-terminal segment, termed the B-upstream segment (BUS), that is not present in the truncated isoform, PR-A (94 kDa). As reported above, we have created a CD domain mutant PR (mCD PR) of PR-B. To identify possible protein interactions that may be disrupted upon mutation of this domain, we used co-immunoprecipitation (Co-IP) assays to screen for putative interacting proteins. We tested the ability of mCD PR to interact with MKP3, a protein previously shown to interact with ERK2 through an identical CD domain [11]. Using COS cells that had been transiently transfected with wt PR-B (hereby referred to as wt PR) or mCD PR, as well as myc-tagged MKP3, we showed that while wt PR interacts with MKP3 both in the presence and absence of ligand, mCD PR failed to interact with MKP3 (Fig 1). Subsequent biochemical experiments using various PR mutants showed how critical the CD domain is in facilitating the PR-MKP3 interaction. The CD domain is located in the BUS region that is unique to PR-B, therefore, PR-A lacks the CD domain. Moreover, we hypothesized that PR-A would not participate in protein complexes whose formation was dependent on the presence of the CD domain. Using COS cells transiently transfected with PR-A (lacking the CD domain), we showed that the CD domain is critical for PR-MKP3 protein complex formation (Fig 2). The interaction between PR-A (lacking the CD domain) and MKP3 is significantly compromised when compared to wt PR (PR-B) (compare lanes 2 and 5). When an artificial CD domain is added onto the N-terminus of PR-A (creating CD-PR-A; lane 6), this artificial PR construct regains the ability to interact with MKP3; an effect that is reversed upon mutation of the artificial CD domain (mCD-PR-A; lane 7). These experiments strongly suggest that the CD domain is critical to facilitating the interaction between wt PR and MKP3.

Co-IP experiments studying a putative interaction between PR and ck2 have thus far been unsuccessful due to limitations in the ability to overexpress ck2. We continue to troubleshoot these experiments, however, the CD domain does not contain sequences known to facilitate interactions between ck2 and its respective substrates, suggesting that a putative interaction between PR and ck2 may be indirect. Co-IPs between PR and other members of the MKP or MEK family have not been tested. These data indicate that PR interacts with MKP3 in a CD domain-dependent manner.

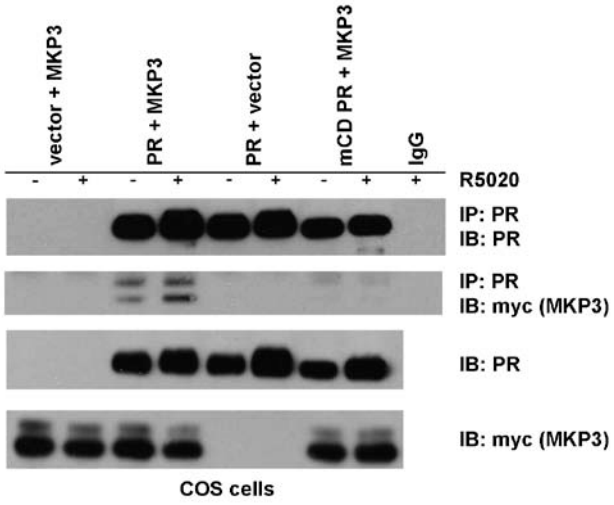

Figure 1. mCD PR fails to interact with MKP3. COS cells were co-transfected with wt or mCD PR, myc-MKP3 or respective vector controls. Following a 24 hr incubation in serum-free media, cells were treated with EtOH or 10nM R5020 for 60 min. Cell lysates were immunoprecipitated with a PR antibody, and the resulting co-immunoprecipitated protein complexes were analyzed by Western blotting (top two panels). Bottom two panels represent total cell lysates.

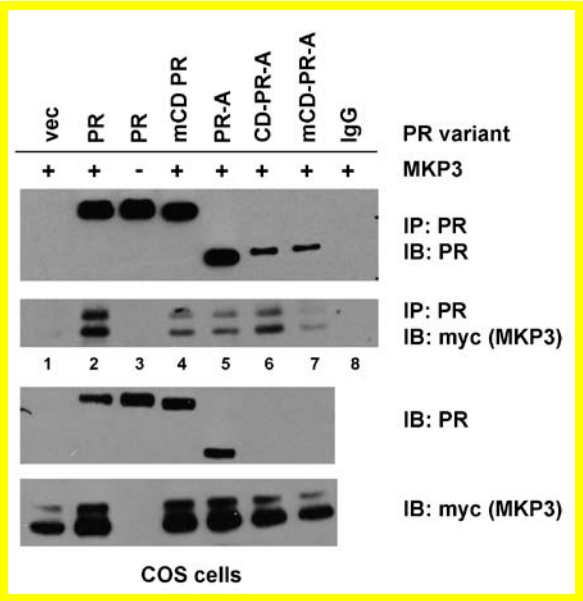

Figure 2. PR CD domain mediates interaction with MKP3. COS cells were co-transfected with myc-MKP3 (or vector control) and one of the following PR constructs: vector (lane 1), wt PR-B (lanes 2-3), mCD PR (lane 4), PR-A (lane 5), CD-PR-A (PR-A with a CD domain attached to the N-terminus; lane 6), or mCD-PR-A (PR-A with a mutant CD domain attached to the N-terminus; lane 7). Lysates were isolated following a 24 hr incubation in serum-free media. Cell lysates were immunoprecipitated with a PR antibody, and the resulting co-immunoprecipitated protein complexes were analyzed by Western blotting (top two panels). Bottom two panels represent total cell lysates.

***Task 2*: Analysis of PR phosphorylation sites that are altered by CD domain interactions (Months 1-12):**

The phosphorylation status of mCD PR in response to ligand was analyzed using phospho-specific PR antibodies. HeLa cells were transiently transfected with wt or mCD PR, and PR phosphorylation in response to ligand was analyzed by Western blotting using antibodies directed to PR Sers 294, 345 and 400 (Fig 3). Interestingly, mCD PR appears to be phosphorylated on an earlier time course as compared to wt PR, with R5020-induced phosphorylation occurring earlier in cells transfected with mCD PR. In contrast, when measuring levels of Ser81 phosphorylation, mCD PR is not phosphorylated on this site in response to ligand (Fig 3). These data suggest that mutation of the CD domain differentially affects PR phosphorylation in a site-specific manner: some sites show hyper-phosphorylation (perhaps due to an altered interaction with a phosphatase, like MKP3 – see Fig 1), whereas other newly characterized PR phosphorylation sites (Ser81; see Appendix A) show decreased phosphorylation in response to ligand, indicating an impaired interaction with a putative PR-modifying kinase, like ck2 (the kinase preliminarily shown *in vitro* to phosphorylate PR on Ser81) [7, 13].

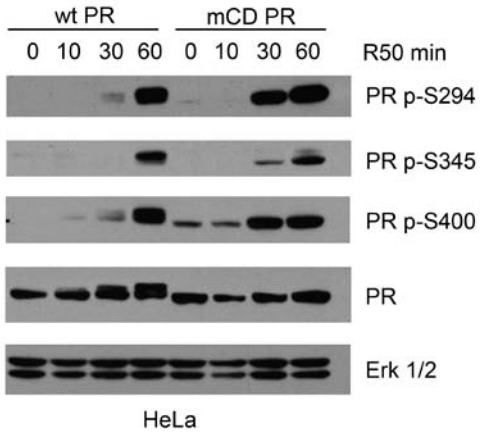

Figure 3. Earlier time-course for progesterone-induced phosphorylation of mCD PR as compared to wt PR. HeLa cells were transfected with either wt or mCD PR. Following transfection, cells were starved for 24 hr in serum-free media and then treated with 10nM R5020 for 0-60 min. Total cell lysates were analyzed by Western blotting.

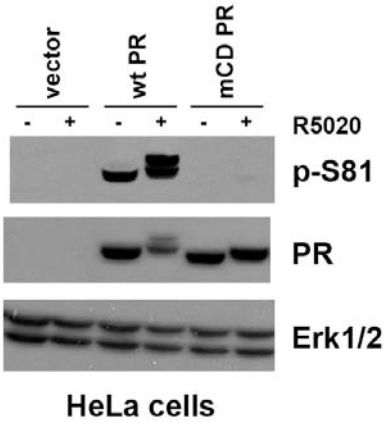

Figure 4. mCD PR lacks phosphorylation on Ser81. HeLa cells were transfected with either wt or mCD PR. Following transfection, cells were starved for 24 hr in serum-free media and then treated with vehicle (EtOH) or 10nM R5020 for 60 min. Total cell lysates were analyzed by Western blotting.

To determine if Ser81 phosphorylation is dependent on phosphorylation at other sites within PR (i.e. pre-requisite phosphorylation needed to subsequently obtain Ser81 phosphorylation), we analyzed S81 phosphorylation of previously characterized PR-phosphorylation mutants (S294A, S345A and S400A). Ser81 phosphorylation was measured in HeLa cells that were transiently transfected with various PR phosphorylation mutants (alanine mutants for serine phosphorylation at Sers 294, 345 and 400). Each of these PR phosphorylation mutants retained the ability to get phosphorylation on Ser81, indicating that phosphorylation at Sers 294, 345 or 400 is not required to obtain Ser81 phosphorylation (Fig 5).

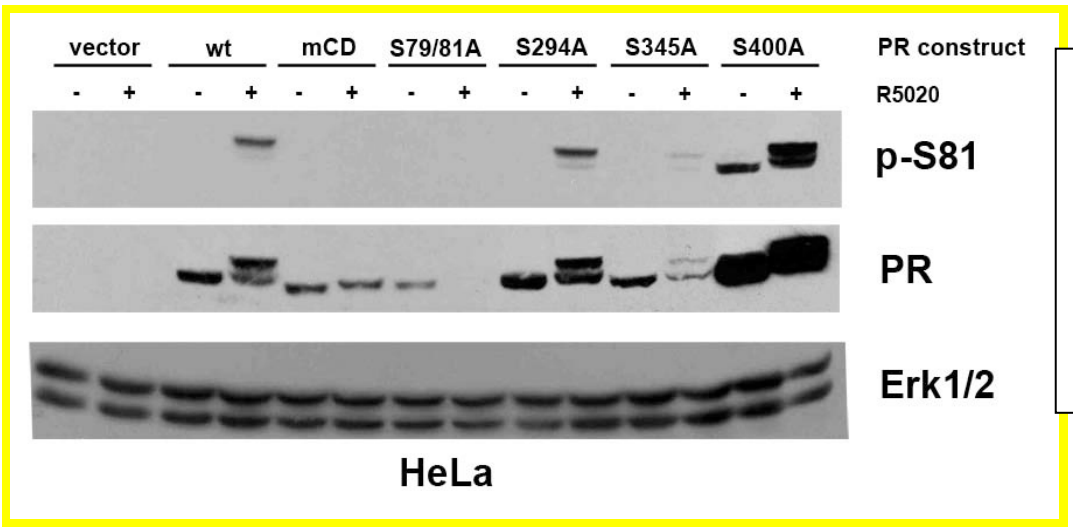

Figure 5. Ser81 phosphorylation independent of other PR phosphorylation. HeLa cells were transfected with the respective PR construct as labeled. Following transfection, cells were starved for 24 hr in serum-free media and then treated with vehicle (EtOH) or 10nM R5020 for 60 min. Total cell lysates were analyzed by Western blotting.

To characterize PR phosphorylation by ck2, the kinase previously shown *in vitro* to phosphorylate PR on Ser81 [7], we analyzed ligand-activated PR phosphorylation in the presence of two highly-specific, synthetic ck2 kinase inhibitors, TBB and DMAT. Data from two different cell lines stably expressing wt PR, HeLa-PR and T47Y-YB, showed that treatment with both inhibitors significantly decreased phosphorylation of Ser81 in response to ligand (Appendix A; Fig 2A-C). In addition to ligand, we found that Ser81 phosphorylation was differentially activated during specific phases of the cell cycle, independent of ligand. Ser81 was phosphorylated in the G1/S phase of the cell cycle (in the absence of ligand); an effect shown to be dependent on ck2 (Appendix A; Fig 2D). We have not yet analyzed the effect of ck2 knockdown (using si/shRNA technology) on Ser81 phosphorylation, but predict that the outcome will be similar to using synthetic kinase inhibitors. These data indicate that PR phosphorylation on Ser81 is regulated by ck2, both in response to ligand and in a cell cycle-dependent manner [13].

***Task 3*: Analysis of CD domain-dependent PR transcriptional activity (Months 6-18):**

Although we have been technically unsuccessful in measuring PR transcriptional activity via PRE-luciferase assays in the presence of ck2 inhibitors (long term inhibition of ck2, as is necessary to measure PR transcriptional products by luciferase, proved to be toxic to both HeLa-PR and T47D-YB cells), we have focused on studying the downstream consequence of ck2 kinase action: phosphorylation on PR Ser81 (thoroughly characterized in Appendix A; [13]). To study the functional significance of PR phosphorylation at this site, we created a PR mutant that cannot get phosphorylated by ck2 by mutating Ser81 to alanine (S79/81A PR). Point mutation of phosphorylated residues within phospho-proteins can shift specificity to adjacent or very nearby phospho-acceptor sites that are not detected using mass spectrometry of the wt protein [14]. Thus, both PR residues (Ser79 and Ser81) were mutated to ensure that nearby Ser79 is not weakly targeted by highly active kinases (*in vivo*) when Ser81 is mutated. Phospho-Ser81 PR antibody specificity was verified using the double phospho-mutant receptor (S79/81A PR). The S79/81A PR mutant does not get phosphorylated on Ser81, but retains functional transcriptional activity as measured by PRE-luciferase (Appendix A, Fig 3B). Stable cell lines were created using this mutant and were used for subsequent experiments (Appendix A, Fig 4). Specifically, T47D-S79/81A PR cells were used to measure transcription of endogenous PR target genes. We found that Ser81 PR phosphorylation regulated transcription in a ck2-dependent manner of a subset of PR target genes known to be involved in cell growth and prevention of apoptosis, including BIRC3, HSD11β2 and HbEGF (Appendix A, Figs 5-7). ChIP and re-ChIP experiments (Appendix A, Fig 8) showed that Ser81 phosphorylation was required for PR recruitment to these genes, both basally (BIRC3, HSD11B2) and in response to ligand (HbEGF).

Analysis of PR target gene transcription in cells stably expressing mCD PR yielded results suggesting that the CD domain regulates known PR-target genes critical to cell growth and mammary stem cell maintenance (STAT5A and Wnt1; Fig 6). T47D cells stably transfected with mCD PR showed significant defects in activating transcription of STAT5A and Wnt1 in response to ligand as compared to wt PR (Fig 6). Activation of these target genes in mCD PR-expressing cells mimicked what was seen in cells stably expressing S79/81A PR, reinforcing the link between the CD domain and Ser81 phosphorylation. Interestingly, basal levels of STAT5A were also affected similarly by mutation of the CD domain and disruption of Ser81 phosphorylation. Both mCD PR and S79/81A PR-expressing cells showed decreased basal (in the absence of ligand) levels of STAT5A transcription (Fig 7), indicating that phosphorylation at Ser81 (facilitated by the CD domain) is required to maintain PR-dependent transcription of STAT5A.

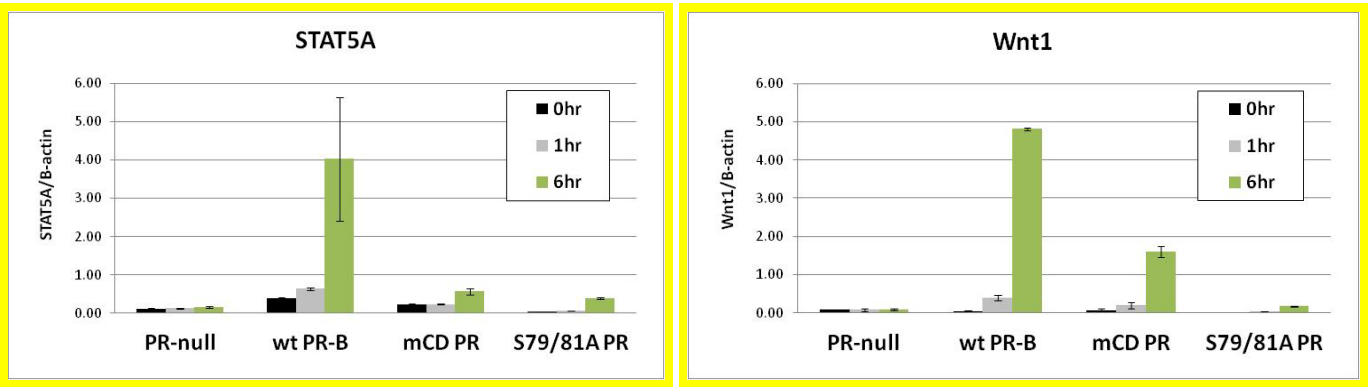

Figure 6. Cells expressing mutant CD or S79/81A PR displayed impaired transcriptional responses.
Stable T47D breast cancer cells expressing wt, mCD or S79/81A PR (or PR-null) were treated for 0-6hr with R5020. mRNA levels were analyzed by qPCR using primers specific to STAT5A (left), Wnt1 (right) or b-actin (internal control).

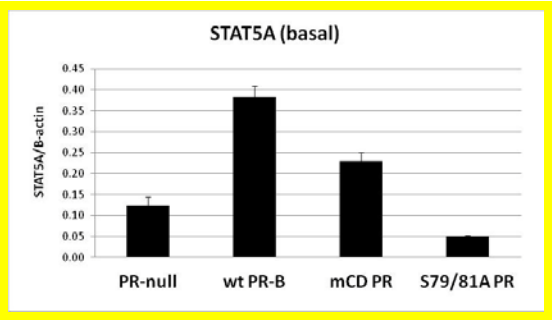

Figure 7. CD domain required for PR-regulated STAT5A basal transcription.
mRNA levels were analyzed by qPCR using primers specific to STAT5A in stable T47D breast cancer cells expressing wt, mCD or S79/81A PR (or PR-null). B-actin mRNA was used as an internal control.

Finally, transcription of MKP3, both basally and in response to ligand, appears to be altered in mCD PR-expressing cells. In the absence of ligand, MKP3 mRNA (Fig 8 – left) and protein (not shown) levels are significantly elevated in mCD PR cells as compared to cells expressing wt PR. Moreover, in response to ligand, MKP3 levels are repressed in wt PR-expressing cells, an effect that is impaired in cells expressing mCD PR (Fig 8 - right; 0-18hr). Interestingly, this phenotype (altered basal and ligand-dependent transcription in mCD PR cells) is not shared by the S79/81A expressing cells, which behave similarly to wt PR-expressing cells. These data indicate that the transcriptional control of MKP3 is independent of Ser81 phosphorylation, and is regulated by the CD domain through a mechanism that has yet to be defined. Experiments are underway to further characterize this phenotype, and to determine the connection between MKP3 protein binding through the CD domain (Figs 1-2) and subsequent regulation of MKP3 mRNA/protein levels (Fig 8). This is an interesting experimental result that we are eager to analyze.

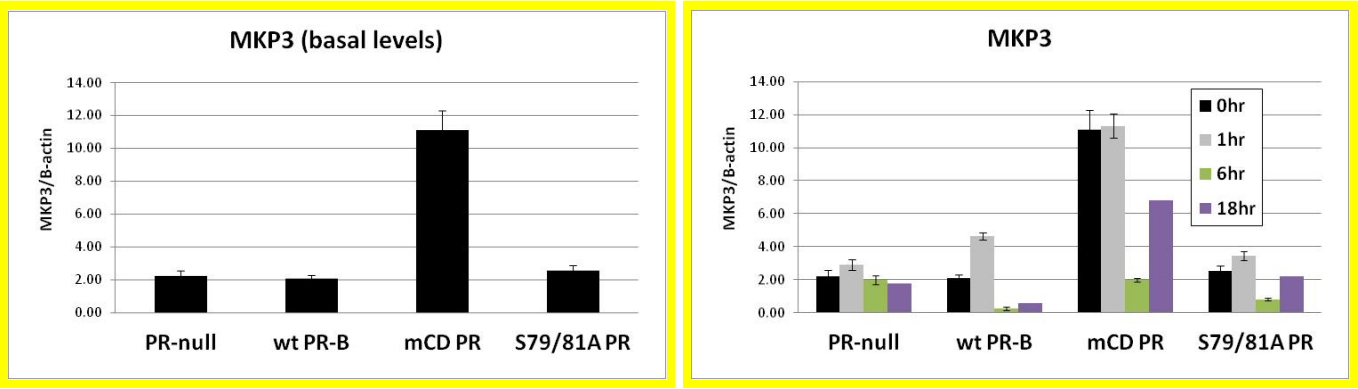

Figure 8. PR CD domain required for PR-regulated MKP3 transcription.
Left: mRNA levels were analyzed by qPCR using primers specific to MKP3 in stable T47D breast cancer cells expressing wt, mCD or S79/81A PR (or PR-null). B-actin mRNA was used as an internal control. Right: Stable T47D breast cancer cells expressing wt, mCD or S79/81A PR (or PR-null) were treated for 0-18hr with R5020. mRNA levels were analyzed by qPCR using primers specific to MKP3 or B-actin (internal control).

Cumulatively, these data suggest that many (STAT5A, Wnt1) CD domain-dependent transcriptional targets overlap with those mediated by S81 phosphorylation, as one primary function of the CD domain appears to be facilitating phosphorylation at Ser81. These CD and phospho-Ser81-dependent genes are known PR-target genes that regulate cell growth and proliferation basally and in response to ligand. Interestingly, we have identified a role for the CD domain that is independent of its Ser81 phosphorylation-associated function: MKP3 mRNA and protein regulation.

***Task 4*: Analysis of CD domain-dependent rapid signaling events (Months 6-12):**

Following treatment with ligand, PR has been shown to rapidly activate (within 15 min) protein kinases, such as MAPK (Erk1/2), c-Src and Akt. To determine if the CD domain of PR is necessary for this function, we transfected HeLa cells with wt and mCD PR constructs and measured MAPK phosphorylation following short

treatments with R5020 (Figure 9). Preliminary experiments suggest that wt and mCD PR similarly activate phosphorylation of Erk1/2, suggesting that the CD domain is not required for this effect. Rapid activation of c-Src and Akt have not been tested. Experiments to test the ability of mutant S79/81A PR to rapidly activate cellular kinases have not yet been initiated, but we would expect the results to be similar to those obtained with mCD PR.

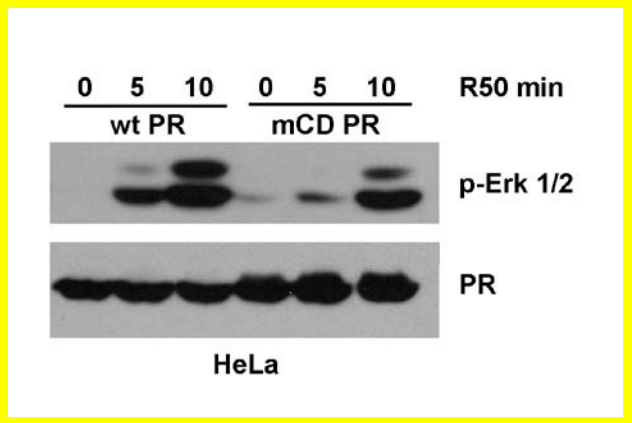

Figure 9. MAPK activation by mCD PR.
HeLa cells were transfected with either wt or mCD PR. Following transfection, cells were starved for 24 hr in serum-free media and then treated with 10nM R5020 for 0-10 min. Total cell lysates were analyzed by Western blotting.

***Task 5*: Analysis of the effect of PR's CD domain on cell proliferation (Months 12-30):**

Using stable cell lines that express wt, mCD or S79/81A PR, preliminary experiments were conducted to determine if mutation of the CD domain or phosphorylation on Ser81 affected cell growth in the presence and absence of ligand. Preliminary data obtained from these experiments suggests that cellular proliferation rates are not affected by the aforementioned mutations, as growth rates are similar amongst the cell lines (data not shown; Appendix A). Cell-cycle specific growth analyses have not yet been performed.

***Task 6*: Analysis of the effect of PR's CD domain on anchorage-independent growth (Months 24-36):**

The ability of mCD PR cells to grown in an anchorage-independent manner has not yet been analyzed. However, these experiments have been conducted with regards to S79/81A PR-expressing cells. Interestingly, cells expressing mutant S79/81A PR, while retaining their ability to grown soft-agar colonies in response to ligand, formed significantly fewer colonies in the ligand-independent condition as compared to cells expressing wt PR (Appendix A, Fig 4C). These data indicate that phosphorylation on Ser81, in the absence of ligand, contributes to cellular survival as measured by anchorage-independent growth.

KEY RESEARCH ACCOMPLISHMENTS

- Task 1 Milestone: MKP3 was identified as a protein that interacts with PR through the CD domain. Mutational studies revealed the critical contribution of the CD domain to facilitating the interaction between PR and MKP3.
- Task 2 Milestone: Ser81 is differentially phosphorylated due to mutation of the PR CD domain; mCD PR lacks phosphorylation at Ser81. Other PR phosphorylation sites studied appear to be hyper-phosphorylated on mCD PR as compared to wt PR.
- Task 2 Milestone: Ser81 phosphorylation occurs independently of other PR site phosphorylation.
- Task 2 Milestone: ck2 is the kinase responsible for phosphorylation of PR on Ser81.
- Task 2 Milestone: Ser81 phosphorylation is regulated basally, in response to ligand, and in a cell cycle-dependent manner; all PR Ser81 phosphorylation is ck2-dependent.
- Task 3 Milestone: A subset of endogenous PR target genes was identified that is regulated by phosphorylation at PR Ser81. This subset contains genes known to regulate cellular proliferation and/or survival.
- Task 3 Milestone: ChIP and re-ChIP experiments confirmed that Ser81 is required for PR recruitment to the aforementioned subset of Ser81-dependent target genes.
- Task 3 Milestone: STAT5A and Wnt1, genes known to be involved in breast cancer cell proliferation and mammary stem cell maintenance, are regulated by PR in a CD domain-dependent manner.
- Task 3 Milestone: Mutation of the CD domain in PR disrupts the transcriptional regulation of MKP3, a protein previously shown to interact with PR through the CD domain; mCD cells have higher levels of MKP3 and are no longer able to transcriptionally repress MKP3 in a PR-dependent manner.
- Task 4 Milestone: The CD domain of PR is not required for PR-dependent rapid activation of MAPK in response to ligand.
- Task 5 Milestone: Cellular proliferation rates are likely not affected by mutations in the CD domain or phosphorylation at Ser81.
- Task 6 Milestone: Phosphorylation at Ser81 regulates the ability of PR-expressing cells to survive in an anchorage-independent manner in the absence of ligand.

REPORTABLE OUTCOMES

- Manuscripts:

  Hagan, C.R., Regan, T.M., Dressing, G.E. and Lange, C.A. ck2-Dependent Phosphorylation of Progesterone Receptors (PR) on Ser81 Regulates PR-B-Isoform-Specific Target Gene Expression in Breast Cancer Cells. ***Mol Cell Biol*** 2011 Jun; 31(12): 2439-2452. (Appendix A)

  Hagan CR, Daniel AR, Dressing GE, Lange CA. Role of phosphorylation in progesterone receptor signaling and specificity. ***Mol Cell Endocrinol.*** 2011 Sep 16 (in press). (Appendix B)

  Daniel AR, **Hagan CR**, Lange CA. Progesterone receptor action: defining a role in breast cancer. ***Expert Rev Endocrinol Metab.*** 2011 May 1;6(3):359-369. (Appendix C)

- Abstracts presented/meetings attended:

  Hagan, C.R., Regan, T.M., Dressing, G.E. and Lange, C.A. ck2-Dependent Phosphorylation of Progesterone Receptors (PR) on Ser81 Regulates PR-B-Isoform-Specific Target Gene Expression in Breast Cancer Cells. 102nd Annual Meeting of the American Association for Cancer Research. April 2-6, 2011.

- Grants submitted:

  K99/R01 NCI research grant submitted 6/2011. Title: CK2-dependent Phosphorylation of Progesterone Receptors Mediates Proliferative Signaling in Breast Cancer. Reviewed 10/2011, Score: 25; decision pending.

CONCLUSION

Progesterone receptors (PR) are critical mediators of mammary gland development and contribute to breast cancer progression. Progestin-induced rapid activation of cytoplasmic protein kinases leads to selective regulation of growth-promoting genes by phospho-PR species. We have shown that phosphorylation of PR Ser81 is ck2-dependent, progestin-regulated and cell cycle-dependent in intact cells. Mutation of the CD domain in PR (mCD PR) abrogates phosphorylation on Ser81, indicating that the CD domain in necessary to facilitate phosphorylation at this site (Ser81). Additionally, we showed that an interaction between PR and MKP3, a regulator of the ERK family, is dependent on the CD domain. Regulation of selected genes by PR-B also required the CD domain for basal and/or progestin-regulated (STAT5A, Wnt1, MKP3) expression/repression. We conclude that the CD domain of PR facilitates protein interactions that are critical to PR-dependent transcription of genes involved in proliferation and mammary stem cell maintenance. Experiments to determine how MKP3 binding mechanistically regulates transcription of these genes, as well as phosphorylation at Ser81 (by ck2), are currently underway. Understanding how mitogenic protein kinases, such as ck2, alter PR phosphorylation and function is critical to fully understanding breast tumor etiology and developing better targeted therapies. Recent clinical data linking the progesterone component of hormone-replacement therapy regimens with the development of breast cancer underscores the importance of understanding how PR works in the context of breast cancer and high kinase environments. Due to the ubiquitous nature of ck2 and its prevalence in many different types of cancer, there has been much interest in the development of ck2 inhibitors as anti-cancer agents. Clinical ck2 inhibitors, in combination with more specific anti-progestins (new classes of selective progesterone receptor modulators or SPRMs), could provide an effective combination of targeted therapy for breast cancer treatment.

References

1. Daniel, A.R., E.J. Faivre, and C.A. Lange, *Phosphorylation-dependent Antagonism of Sumoylation De-represses Progesterone Receptor Action in Breast Cancer Cells.* Mol Endocrinol, 2007.
2. Lange, C.A., T. Shen, and K.B. Horwitz, *Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome.* Proc Natl Acad Sci U S A, 2000. **97**(3): p. 1032-7.
3. Weigel, N.L., et al., *Phosphorylation and progesterone receptor function.* J Steroid Biochem Mol Biol, 1995. **53**(1-6): p. 509-14.
4. Ward, R.D. and N.L. Weigel, *Steroid receptor phosphorylation: Assigning function to site-specific phosphorylation.* Biofactors, 2009. **35**(6): p. 528-36.
5. Meggio, F. and L.A. Pinna, *One-thousand-and-one substrates of protein kinase CK2?* FASEB J, 2003. **17**(3): p. 349-68.
6. Tawfic, S., et al., *Protein kinase CK2 signal in neoplasia.* Histol Histopathol, 2001. **16**(2): p. 573-82.
7. Zhang, Y., et al., *Identification of phosphorylation sites unique to the B form of human progesterone receptor. In vitro phosphorylation by casein kinase II.* J Biol Chem, 1994. **269**(49): p. 31034-40.
8. Beral, V., *Breast cancer and hormone-replacement therapy in the Million Women Study.* Lancet, 2003. **362**(9382): p. 419-27.
9. Anderson, G.L., et al., *Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial.* JAMA, 2004. **291**(14): p. 1701-12.
10. Rubinfeld, H., T. Hanoch, and R. Seger, *Identification of a cytoplasmic-retention sequence in ERK2.* J Biol Chem, 1999. **274**(43): p. 30349-52.
11. Tanoue, T., et al., *A conserved docking motif in MAP kinases common to substrates, activators and regulators.* Nat Cell Biol, 2000. **2**(2): p. 110-6.
12. Burgermeister, E., et al., *Interaction with MEK causes nuclear export and downregulation of peroxisome proliferator-activated receptor gamma.* Mol Cell Biol, 2007. **27**(3): p. 803-17.
13. Hagan, C.R., et al., *ck2-Dependent Phosphorylation of Progesterone Receptors (PR) on Ser81 Regulates PR-B Isoform-Specific Target Gene Expression in Breast Cancer Cells.* Mol Cell Biol, 2011. **31**(12): p. 2439-52.
14. Resing, K.A., et al., *Determination of v-Mos-catalyzed phosphorylation sites and autophosphorylation sites on MAP kinase kinase by ESI/MS.* Biochemistry, 1995. **34**(8): p. 2610-20.

Molecular and Cellular Biology

ck2-Dependent Phosphorylation of Progesterone Receptors (PR) on Ser81 Regulates PR-B Isoform-Specific Target Gene Expression in Breast Cancer Cells

Christy R. Hagan, Tarah M. Regan, Gwen E. Dressing and Carol A. Lange

Mol. Cell. Biol. **2011, 31(12):2439. DOI: 10.1128/MCB.01246-10.**
Published Ahead of Print 25 April 2011.

MOLECULAR AND CELLULAR BIOLOGY, June 2011, p. 2439–2452 Vol. 31, No. 12
0270-7306/11/$12.00 doi:10.1128/MCB.01246-10

ck2-Dependent Phosphorylation of Progesterone Receptors (PR) on Ser81 Regulates PR-B Isoform-Specific Target Gene Expression in Breast Cancer Cells[▽]

Christy R. Hagan, Tarah M. Regan, Gwen E. Dressing, and Carol A. Lange*

Department of Medicine and Women's Cancer Program, University of Minnesota, Minneapolis, Minnesota

Received 27 October 2010/Returned for modification 22 December 2010/Accepted 11 April 2011

Progesterone receptors (PR) are critical mediators of mammary gland development and contribute to breast cancer progression. Progestin-induced rapid activation of cytoplasmic protein kinases leads to selective regulation of growth-promoting genes by phospho-PR species. Herein, we show that phosphorylation of PR Ser81 is ck2 dependent and progestin regulated in intact cells but also occurs in the absence of PR ligands when cells enter the G_1/S phase of the cell cycle. T47D breast cancer cells stably expressing a PR-B mutant receptor that cannot be phosphorylated at Ser79/81 (S79/81A) formed fewer soft agar colonies. Regulation of selected genes by PR-B, but not PR-A, also required Ser79/81 phosphorylation for basal and/or progestin-regulated (BIRC3, HSD11β2, and HbEGF) expression. Additionally, wild-type (wt) PR-B, but not S79/81A mutant PR, was robustly recruited to a progesterone response element (PRE)-containing transcriptional enhancer region of BIRC3; abundant ck2 also associated with this region in cells expressing wt but not S79/81A PR. We conclude that phospho-Ser81 PR provides a platform for ck2 recruitment and regulation of selected PR-B target genes. Understanding how ligand-independent PRs function in the context of high levels of kinase activities characteristic of breast cancer is critical to understanding the basis of tumor-specific changes in gene expression and will speed the development of highly selective treatments.

The ovarian steroid hormone progesterone acts by binding to and activating progesterone receptor (PR) A, B, and C isoforms expressed in target tissues. In the normal breast, PR-A and PR-B are typically expressed in a minority population (7 to 10%) of luminal epithelial cells. PR-B is required for mammary gland development during puberty and pregnancy and acts by contributing to lobulo-alveolar proliferation and ductal side branching (8, 46). Studies from PR-knockout mice show that these mice have significant defects in mammary gland morphology (primarily PR-B dependent) and reproductive abnormalities (primarily PR-A driven) (46, 54). Additionally, the presence of PR was shown to be required for the formation of mammary tumors in a carcinogen-induced mouse model of breast cancer (47). Finally, recent clinical data have shown that women taking hormone replacement therapy (HRT) whose regimens included both estrogen and a progestin, but not estrogen alone, experienced increased breast tumor numbers and sizes (1, 5, 12). Interestingly, the effect of combined HRT on breast cancer risk was reversible (5, 13), suggestive of epigenetic events.

In the absence of progesterone, PR molecules rapidly shuttle between the cytoplasm and the nucleus; cytoplasmic PRs contain membrane-associated species capable of direct binding and signaling to mitogenic protein kinases (c-Src, MAPK, PI3K) (3, 7, 25, 50). Following ligand binding, PRs dissociate from heat shock protein-containing chaperone complexes, undergo dimerization, and are largely retained in the nucleus. Nuclear receptors activate transcription of PR target genes, either directly through binding to progesterone response elements (PREs) or indirectly through tethering interactions with other transcription factors (AP1, SP1, STATs) (14, 61, 70). Notably, PR is highly posttranslationally modified, primarily on serine (phosphorylation) and lysine (acetylation, ubiquitination, and sumoylation) residues located in the N-terminal region (16, 17, 43, 76). These modifications are frequently ligand dependent but can also occur independently of progestin binding and significantly alter receptor stability, localization, tethering interactions, transcriptional activity, and promoter selectivity (18, 75). For example, MAPK and cdk2 have previously been shown to phosphorylate and modulate the activity of both liganded and unliganded PR (43, 62, 79).

The serine-threonine protein kinase ck2 (formerly casein kinase II) is ubiquitously expressed with over 300 substrates, many of which are involved in proliferation, cell survival, and gene expression (49). Moreover, ck2 has been shown to be overexpressed in many different types of cancer, including breast cancer (31). ck2, a holoenzyme composed of two catalytic subunits (α and α′) and two regulatory subunits (β), is a unique kinase in that it is constitutively active and does not require modifications or signaling inputs to modulate its kinase activity. In contrast, one mode of ck2 regulation likely occurs via altered subcellular localization of ck2 and/or its respective substrates (27). ck2 localization appears to be altered in a cell cycle-dependent manner, with nuclear accumulation occurring primarily in G_1/S (51, 78). However, subcellular sequestration is not the only proposed mechanism for ck2 regulation. Others include regulated assembly of the ck2 holoenzyme, protein complex formation with substrates, autophosphorylation, and

* Corresponding author. Mailing address: Departments of Medicine (Hematology, Oncology, and Transplantation) and Pharmacology, University of Minnesota Masonic Cancer Center, 420 Delaware St. SE, MMC 806, Minneapolis, MN 55455. Phone: (612) 626-0621. Fax: (612) 626-4915. E-mail: lange047@umn.edu.

▽ Published ahead of print on 25 April 2011.

small molecule interactions (59); little is known about this topic.

Understanding how a cancer-associated kinase, like ck2, modulates PR function may provide insight into how PR promotes breast cancer cell proliferation (a PR-B-dependent action) and tumor progression (31). ck2 has previously been shown *in vitro* to phosphorylate human PR at Ser81, a residue located in the N-terminal region of PR unique to PR-B, termed the B-upstream segment (BUS) (80). Subsequent *in silico* analysis revealed 11 potential ck2 phosphorylation sites in PR (80). Mass spectrometry studies and *in vitro* kinase assays revealed that Ser81 was the primary site for ck2 phosphorylation; these studies failed to detect phosphorylation on any of the other ck2 consensus sites in PR (80). However, these studies were done using solely *in vitro* model systems; regulated phosphorylation at this site has not been studied in intact cells. Herein, we sought to understand the functional significance of ck2 regulation of PR-B Ser81 in breast cancer models.

MATERIALS AND METHODS

Cell lines. The estrogen-independent ER/PR positive T47Dco (T47D) variant cell line has been previously described (35). T47D-Y (PR negative), T47D-YB (stably expressing wild-type [wt] PR-B), and T47D-YA (stably expressing wt PR-A) cells were characterized by Sartorius et al. (66). HeLa-PR cells have been previously described (62). T47D-S79/81A PR cells were created by stable expression of pSG5-S79/81A PR and pSV-neo in T47D-Y cells using FuGene-HD (Roche). Individual colonies were selected in 500 μg/ml G418 and maintained in 200 μg/ml G418 after initial selection. The pSG5-S79/81A PR plasmid (containing serine-to-alanine mutations at Ser79 and Ser81) was generated by GenScript Corporation. T47D-Y and HeLa cells were maintained at 37°C in 5% CO_2 in minimum essential media (MEM) (CellGro) supplemented with 5% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% nonessential amino acids, and 6 ng/ml insulin (cMEM). T47D-YB, T47D-YA, T47D-S79/81A PR, and HeLa-PR cells were maintained under the same conditions, with the addition of 200 μg/ml G418.

T47D cells containing an inducible PR expression system were created as follows using the ARGENT regulated transcription retrovirus kit (ARIAD Pharmaceuticals, Inc.). T47D-Y cells were first stably retrovirally transduced with the transcription factor vector pL_2N_2-R_HS3H/ZF3 (necessary for activating subsequent transcription from the target gene vector). A clone from this cell line was stably retrovirally transduced with the target gene vector (pLH-Z_{12}I-PL) containing wt PR-B (iPR-B) or with the empty vector (iEV). Upon addition of a chemical dimerizer (AP21967; 10^{-9} M), PR-B protein expression occurs within 24 to 48 h (as measured by Western blotting). These cells are maintained in cMEM supplemented with 200 μg/ml G418 and hygromycin B (CalBioChem).

Transient-transfection experiments were performed as follows: 24 h after cell plating, HeLa cells were transfected with pSG5-vector, pSG5-wt PR or pSG5-S79/81A PR using FuGene6 (Roche). At 24 h following transfection, cells were starved for 18 h in serum-free iMEM (modified improved MEM). Following starvation, cells were treated as noted in the respective figure legend and total cell lysates were isolated as described below.

Immunoblotting. For most of the immunoblotting presented here (exceptions noted in figure legends), cells were starved for 18 h in serum-free iMEM. Following 18 h starvation, cells were treated, if applicable. Whole-cell lysates were isolated using a modified radioimmune precipitation assay (RIPA) buffer (0.15 M NaCl, 6 mM Na_2HPO_4, 4 mM NaH_2PO_4, 2 mM EDTA, 1% Triton-X, 0.1 M NaF; in H_2O) supplemented with protease and phosphatase inhibitors. Lysates containing equal protein levels (between 25 and 30 μg protein was loaded per lane on each gel) were separated by SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore) for subsequent immunoblotting analysis. Membranes were probed with primary antibodies recognizing total PR (number MS-298-P; ThermoScientific), phospho-Ser294 (MS-1332; Lab Vision Corp.), Erk1/2 (9102; Cell Signaling), phospho-Erk1/2 (9101; Cell Signaling), ck2α (sc-12738; Santa Cruz Biotechnology), and ck2β (sc-12739; Santa Cruz Biotechnology). The phospho-Ser81 (p-S81) PR antibody was a custom antibody commissioned from Invitrogen designed to recognize the following phospho-specific peptide sequence (PR-B amino acids 76 to 85): DQQSL-pS-DVEG. Mouse and rabbit horseradish peroxidase-conjugated secondary antibodies were obtained from Bio-Rad, and chemiluminescence was visualized using SuperSignal West Pico chemiluminescent substrate (Pierce Chemical Company). All Western blotting experiments were performed at a minimum in triplicate, and representative experiments are shown in each respective figure.

Luciferase transcription assays. Luciferase assays were performed as previously described (25) using the dual luciferase reporter assay (Promega). Relative luciferase units (RLU) were normalized to the mean result ± standard deviation (SD) for *Renilla* luciferase.

Reagents. Cells were treated with the following reagents (when applicable): R5020 (10 nM; Sigma), RU486 (100 nM; Sigma), EGF (30 ng/ml; Sigma), TBB (1 to 100 μM; CalBioChem), DMAT (1 to 100 μM; CalBioChem), PP2 (10 μM; CalBioChem), roscovitine (100 μM; CalBioChem), U0126 (10 μM; CalBioChem), and AP21967 (1 nM; ARIAD Pharmaceuticals, Inc.).

Cell cycle analysis/flow cytometry. A total of 1.5×10^5 T47D-YB cells were plated in 10-cm^2 dishes in cMEM (day 0). Synchronized cells were treated on day 1 with cMEM containing 2.5 μg/ml thymidine (Sigma) for 18 h. Cells were then washed with phosphate-buffered saline (PBS) and fresh iMEM-5% dextran-coated charcoal (DCC)-treated serum was added for 7 h. Synchronized cells were then treated for 18 h with iMEM-5% DCC-50 μg/ml mimosine. Following the 18-h mimosine treatment (and, if applicable, 60 min treatment with vehicle or TBB), cells were harvested in RIPA for Western blotting (as above) or trypsinized and fixed for flow cytometry. For flow cytometry analysis, media and wash (2 ml PBS) were collected. Trypsinized cells and collected media/wash were combined and pelleted by centrifugation. Cells were resuspended in 300 μl PBS-10% FBS, following which 4 ml ice cold 80% ethanol was added dropwise to fix samples. Samples were stored at −20°C until analyzed for cell cycle phase. Fixed cells were pelleted and washed three times with 5 ml cold PBS. Samples were resuspended in 100 to 400 μl staining buffer: 1× PBS with 10% RNase A (10 mg/ml Sigma), 5% FBS, 0.5 mM EDTA, 0.1% TX-100, and 200 μg/ml propidium iodide (Sigma). Propidium iodide staining was detected using a FACSCalibur (BD Biosciences). Cells were gated for cell cycle phase using FlowJo (Tree Star Inc.).

Soft agar anchorage-independent growth assays. Soft agar assays were performed as previously described (16). Briefly, cells were suspended in 0.48% SeaPlaque GTG agarose (Lonza) in iMEM supplemented with 5% DCC serum containing either ethanol (EtOH) or 10 nM R5020. Cells were plated in triplicate/condition at 9.6×10^3/well over a bottom layer of 0.8% agarose/iMEM with 5% DCC serum. Cells were incubated under normal growth conditions for 21 days, following which colonies were counted in 15 fields/treatment group. The data are represented as an average number of colonies per field ± standard error of the mean (SEM). Soft agar experiments were performed in triplicate.

qPCR. Cells were plated at 5×10^5 cells/well in triplicate wells of a 6-well plate. Following 18 h starvation in serum-free iMEM, cells were treated for 1 to 18 h with 10 nM R5020 or EtOH (if applicable; see relevant figure legend). Total RNA was isolated using Trizol (Invitrogen); cDNA was created using the Transcriptor cDNA first-strand cDNA synthesis kit (Roche) by following the manufacturer's recommendations. Real-time quantitative PCR (qPCR) was performed on equal amounts of cDNA using the Light Cycler 480 SYBR Green1 master mix on a Roche 480 light cycler. Results in triplicate for each gene of interest were normalized to those for either β-actin, 18S, or GAPDH (as indicated in each respective graph) ± SD.

For qPCR experiments on G_1/S synchronized cells, cells were plated at 2.5×10^5/well in triplicate wells of a 6-well plate. Cells were synchronized as described above, and RNA/cDNA was created and analyzed as described above.

ChIP assays. ChIP and ReChIP assays were performed using the ChIP-IT express or Re-ChIP-IT kit (Active Motif), according to the manufacturer's instructions using sonication as the method for chromatin shearing. Lysates were immunoprecipitated (IP) overnight (18 h) with the following antibodies: PR (number MS-298-P; ThermoScientific), ck2α (number sc-12738; Santa Cruz), or an equal amount of mouse or rabbit IgG. Resulting DNA was analyzed using qPCR as described above, and data are represented as a percentage of input DNA. *In silico* analysis using MatInspector (Genomatix) identified potential PRE-binding sites using the following consensus sequence: RGNACANRNTG TNCY. Primer sets used for qPCR analysis of ChIP data are as follows: BIRC3 PRE1-F (5′-AAAACAATAGTGCCAGTTCAATGAC-3′), BIRC3 PRE1-R (5′-ATGTTCTCTTTGATTCCCTGACAC-3′), BIRC3 (neg control 1)-F (5′-TTATGCTGAGCTGGAAGTTAAATAAAAAG-3′), BIRC3 (neg control 1)-R (5′-TTGGCCACTGGTCTCAAACTC-3′), BIRC3 (neg control 2)-F (5′-TGGGAAAAGTGCAGTATTTGG-3′), BIRC3 (neg control 2)-R (5′-GTTCATCTAATTGGGACTGGTTG-3′), TF PRE2-F (5′-TCATTTTAAGACGTCAGCTATTTCAC-3′), TF PRE2-R (5′-ATATTCTCCAGTCAGCATTTCAAAG-3′), TF (neg control 1)-F (5′-CTGAGAATCTATTGGTATTGCTTGG-3′), TF (neg

control 1)-R (5′-CCCTTACGTGAGAAAGTCATTTTG-3′), TF (neg control 2)-F (5′-CTAGATGTGGATGAAATGAGTTGG-3′), and TF (neg control 2)-R (5′-TTCTGAAAGAAAACTAAGCCAAAAC-3′).

Statistics. Statistical significance for all experiments was determined using an unpaired Student's *t* test.

RESULTS

Hormone- and ck2-dependent regulation of PR Ser81 phosphorylation. Previous studies have shown that PR is phosphorylated on Ser81 *in vitro* (80). However, regulation of this site *in vivo* has yet to be defined. Using custom-made polyclonal antibodies created to recognize PR phospho-Ser81, we measured progestin-induced phosphorylation of this site in T47Dco human breast cancer cells (Fig. 1A). T47Dco cells are unmodified breast cancer cells that naturally constitutively express both PR-A and PR-B, without the requirement of estrogen treatment to induce PR expression (35). We detected weak basal PR-B Ser81 phosphorylation that substantially increased in response to treatment with the synthetic progesterone R5020 (Fig. 1A). PR-A does not contain Ser81, located within the BUS domain of PR-B. As expected, our phospho-Ser81-specific antibodies detected PR-B but not PR-A.

In most steroid hormone receptor-positive breast cancer cell models, the levels of PR are primarily upregulated by estradiol, making experimental isolation of PR action (i.e., as studied independently of estrogen) very difficult (34). A naturally occurring PR-negative variant of the T47Dco human breast cancer cell line, termed T47D-Y, was first described by Sartorius and coworkers (66). This parental cell line was used to create stable cell lines constitutively expressing either wild-type (wt) PR-B (T47D-YB) or PR-A (T47D-YA) (66). As observed in unmodified T47Dco cells (Fig. 1A), we also detected low basal levels of Ser81 phosphorylation in T47D-YB cells (Fig. 1B). Again (as in T47Dco cells), the level of PR Ser81 phosphorylation increased significantly in response to R5020 (Fig. 1B). Control cells not expressing PR (T47D-Y) failed to exhibit any nonspecific bands with phospho-S81 or total PR antibodies, indicating a high degree of specificity.

T47D and HeLa cells (stably or transiently expressing PR isoforms) are routinely used as model systems for studying PR action; these cell lines behave similarly with regard to the regulation of posttranslational PR modifications and subsequent changes in receptor function (19, 24, 62). To determine the kinetics of PR Ser81 phosphorylation, we analyzed T47D and HeLa cells stably expressing PR-B. Following a time course of 10 nM R5020 treatment (0 min to 6 h), we observed increased Ser81 phosphorylation beginning at 10 min (T47D-YB) (Fig. 1C) to 15 min (HeLa-PR) (data available on request). This reached a maximum level in both cell lines at 30 to 60 min (Fig. 1C and data available on request). PR Ser81 phosphorylation preceded the ligand-dependent PR upshift primarily mediated by phosphorylation events on one or more unidentified residues (71). Note that ligand-dependent downregulation of PR was observed after at least 4 h of R5020 treatment in both cell lines (58).

PR phosphorylation on Ser294, Ser345, and Ser400 occurs in response to either progestins (i.e., R5020) or mitogenic inputs to MAPKs and/or cdk2 (i.e., EGF, serum) (24, 62, 79). To determine the potential for mitogenic inputs to regulate Ser81 phosphorylation, we performed a time course of EGF treatment in HeLa-PR cells (data available on request). PR Ser81 phosphorylation was not affected by this mitogen, following up to 60 min of EGF treatment, despite significant activation of Erk1/2 over the same time course. To test a broader spectrum

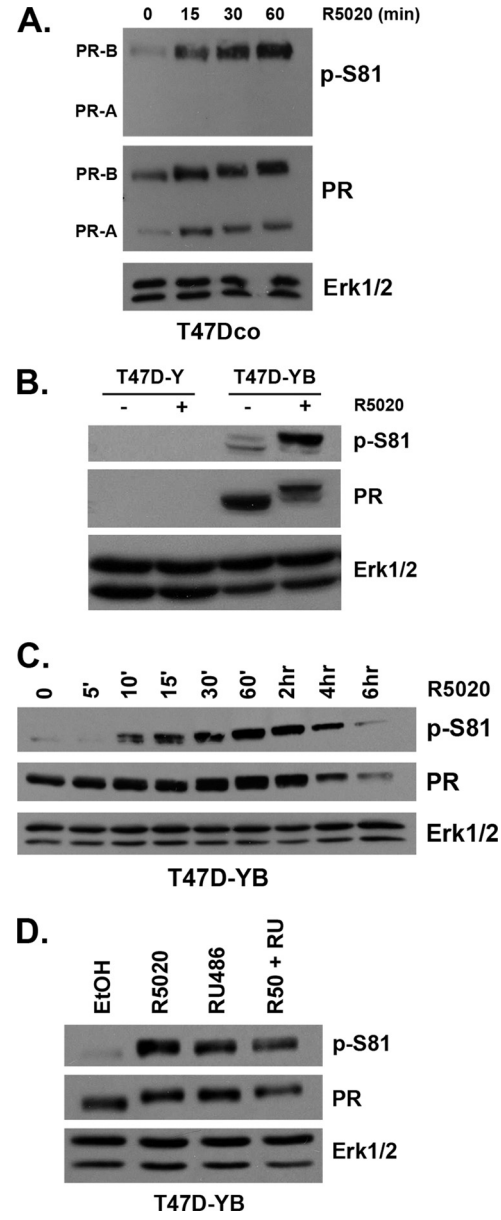

FIG. 1. *In vivo* phosphorylation of PR Ser81. (A) T47Dco cells were starved for 18 h in serum-free media followed by treatment with 10 nM R5020 or ethanol (vehicle) for 0 to 60 min. Lysates were analyzed by Western blotting using antibodies against total Erk1/2 (loading control), total PR, and a custom-designed antibody that specifically recognizes phosphorylated Ser81 PR (p-S81). (B) Cells lacking PR (T47D-Y) and cells stably expressing PR-B (T47D-YB) were serum starved for 18 h and then treated with 10 nM R5020 or EtOH for 60 min. Lysates were analyzed by Western blotting as described for panel A. (C) Following 18 h serum starvation, T47D-YB cells were treated with a time course of 10 nM R5020 for 0 min to 6 h. Lysates were analyzed by Western blotting as described for panel A. (D) Following 18 h serum starvation, T47D-YB cells were treated with 10 nM R5020, 100 nM RU486, both, or vehicle control (EtOH). Lysates were analyzed by Western blotting as described for panel A.

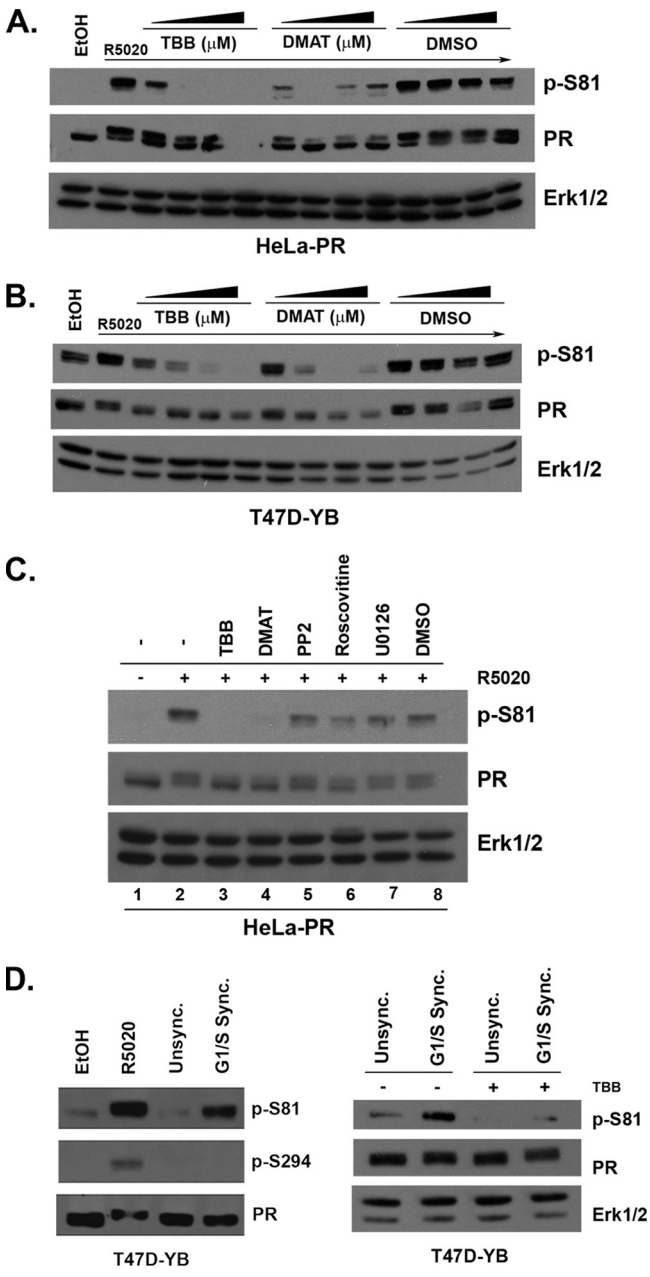

FIG. 2. PR Ser81 is phosphorylated by endogenous ck2. (A and B) HeLa-PR (A) and T47D-YB (B) cells were serum starved for 18 h. Cells were then pretreated with increasing doses of TBB (1 to 100 μM), DMAT (1 to 100 μM), or DMSO (vehicle) for 30 min, followed by 10 nM R5020 for 30 min. Alternatively, cells were treated with R5020 for 30 min or vehicle (EtOH) with no pretreatment. Lysates were analyzed by Western blotting using p-S81, PR, and Erk1/2 antibodies. (C) HeLa-PR cells were starved for 18 h in serum-free medium. Cells were then pretreated (30 min) with TBB (10 μM), DMAT (10 μM), PP2 (10 μM), Roscovitine (100 μM), U0126 (10 μM), or vehicle (DMSO) or left untreated. Following kinase inhibitor pretreatments, cells were treated with 10 nM R5020 or vehicle (EtOH) for 30 min. Lysates were analyzed by Western blotting as described for panel A. (D) Left: T47D-YB cells were serum starved for 18 h and treated with EtOH or 10 nM R5020 for 60 min (left two lanes). Alternatively, cells were treated sequentially as follows: 18 h with thymidine (2.5 μg/ml) or vehicle (PBS), iMEM plus 5% DCC for 7 h, iMEM-5% DCC-mimosine (50 μg/ml; G1/S Sync.) or vehicle (EtOH; Unsync.) for 18 h. Following synchronization (confirmed by flow cytometry; data not shown), protein was analyzed via Western blotting with antibodies for p-S81, phospho-Ser294 (p-S294), or PR. Right: T47D-YB cells were synchronized as just described (or treated with vehicle; Unsync.). Following synchronization, cells were treated for 60 min with vehicle (DMSO) or TBB (10 μM). Protein was analyzed via Western blotting with antibodies for p-Ser81, PR, or Erk1/2 (loading control).

of mitogens, we used fetal bovine serum (FBS; 20%) as a rich source of multiple growth factors. HeLa-PR cells were grown overnight either in serum-free medium, medium supplemented with 5% DCC (charcoal-stripped steroid-free medium), or full growth medium (5% FBS), followed by treatment with either R5020 (positive control for Ser81 phosphorylation; 60 min) or 20% FBS (15 or 60 min). Only R5020 treatment induced robust PR Ser81 phosphorylation (data available on request); no phosphorylation was detected following any of the serum treatments. MAPK (Erk1/2) phosphorylation served as a positive control for serum/mitogenic treatment. Finally, we used the synthetic PR antagonist/partial agonist, RU486, to demonstrate the specificity of PR ligand induction of Ser81 phosphorylation. T47D-YB (Fig. 1D) and HeLa-PR (data available on request) cells were treated with R5020, RU486, or a combination of both. Both ligands induced potent PR Ser81 phosphorylation, while the combination of R5020 plus RU486 was neither additive nor inhibitory. Cumulatively, these data suggest that PR Ser81 phosphorylation occurs primarily in response to progestins, although we frequently observed a low level of basal phosphorylation at this site (see Fig. 1; addressed below).

In vitro kinase assays suggest that ck2, a ubiquitously expressed Ser/Thr protein kinase, directly phosphorylates PR on Ser81 (80). We probed the requirement for ck2 kinase activity in intact cells using two different synthetic, highly specific ck2 kinase inhibitors, TBB and DMAT (23). HeLa-PR and T47D-YB cells were pretreated with increasing concentrations of either TBB or DMAT (or dimethyl sulfoxide [DMSO] vehicle alone) for 30 min, followed by 30 min of R5020. Again, PR Ser81 was potently phosphorylated in response to treatment of cells with R5020 alone (30 min). However, hormone-induced PR Ser81 phosphorylation was completely blocked with either of the ck2 inhibitors in both HeLa-PR (Fig. 2A) and T47D-YB (Fig. 2B) cells. We observed a loss of PR protein at high doses of TBB, the more potent of the two ck2 inhibitors. This is likely due to increased PR degradation, as ck2 is a key regulator of the PR chaperone molecule, hsp90; ck2-mediated phosphorylation of hsp90 is essential for its chaperone activity (52). These data suggest that ck2 kinase activity is required for ligand-dependent PR Ser81 phosphorylation. To determine the specificity of this phosphorylation event *in vivo*, we examined Ser81 phosphorylation in the presence of a broad spectrum of inhibitors for kinases known to affect PR phosphorylation at other N-terminal serine residues, including PP2 (c-Src; Ser345), Roscovitine (cdk2; Ser400), and U0126 (MEK1-MAPK; Ser294) (24, 62, 68). HeLa-PR cells were pretreated with each kinase inhibitor, followed by R5020 for 30 min. Again, Ser81 was robustly phosphorylated in response to R5020. While DMSO alone (the vehicle for each kinase inhibitor) somewhat reduced R5020-induced PR Ser81 phosphorylation (Fig. 2C, compare lane 2 to lane 8), this ligand-regulated phosphorylation event was completely inhib-

ited (compare lane 8 to lanes 3 and 4) only in the presence of the ck2 inhibitors. Together, these data suggest that in the presence of progestin, PR is phosphorylated on Ser81 specifically by (endogenous) ck2.

ck2 has been shown to be regulated in part by cell cycle-dependent localization to the nucleus (51, 78). Steroid receptors rapidly shuttle between the cytoplasm and nucleus; in the presence of progestins, PRs are primarily nuclear. To further address the potential for ck2-mediated regulation of PR Ser81 in the absence of progestins (i.e., basal phosphorylation levels observed above), we tested the cell cycle dependence of this event. For these studies, T47D-YB cells were synchronized at the G_1/S transition using mimosine, a chemical inhibitor of DNA replication; synchronization of control (vehicle) and mimosine-treated T47D-YB cultures was confirmed by flow cytometry (data not shown). In G_1/S-synchronized T47D-YB cells, but not vehicle controls, we observed robust PR Ser81 phosphorylation in the complete absence of ligand (Fig. 2D, left), but it was comparable in magnitude to levels induced following progestin (R5020 or RU486) treatment of unsynchronized cells (Fig. 1D and Fig. 2D, left). Ser294, a MAPK site primarily regulated only in PR-B, was unaffected by mimosine-induced synchronization (Fig. 2D, left). To confirm the ck2 dependence of PR Ser81 phosphorylation in G_1/S phase cells, we treated synchronized populations of cells with or without the ck2 inhibitor, TBB. As in progestin-treated cells above (Fig. 2A to C), ligand-independent PR Ser81 phosphorylation in G_1/S phase cells was completely blocked by addition of the ck2 inhibitor (Fig. 2D, right). Cumulatively, these data suggest that phosphorylation of PR Ser81 occurs independently of ligand when breast cancer cells are passing through the G_1/S phase of the cell cycle, a period when ck2 is primarily nuclear (51, 78). Notably, ck2 is both cytoplasmic and nuclear in untreated T47D cells. Upon progestin-induced nuclear localization of PR, we observed only subtle increases in nuclear relative to cytoplasmic ck2 (data not shown).

PR Ser81-dependent transcriptional activity and promoter selectivity. To investigate the functional consequences of PR Ser81 phosphorylation by ck2, we created a phospho-mutant receptor. Point mutation of phosphorylated residues within phospho-proteins can shift specificity to adjacent or very nearby phospho-acceptor sites that are not detected using mass spectrometry of the wt protein (63). Thus, both PR residues (Ser79 and Ser81) were mutated to ensure that nearby Ser79 is not weakly targeted by highly active kinases (*in vivo*) when Ser81 is mutated. Phospho-Ser81 PR antibody specificity was verified using the double phospho-mutant receptor (S79/81A PR). Western blotting showed that when transiently transfected into HeLa cells, wt PR and S79/81A PR-B were expressed at equal levels; following treatment with R5020, Ser81 phosphorylation was detected only in cells transfected with wt PR (Fig. 3A). Notably, wt and S79/81A receptors were similarly phosphorylated on all other PR phosphorylation sites tested (Ser190, Ser294, Ser345, and Ser400; data not shown), suggesting that mutant receptors fold properly and bind ligand. To determine if phospho-mutant S79/81A PR was capable of binding DNA and subsequently activating transcription, we analyzed wt and mutant PRs using PRE-luciferase reporter gene assays. In transiently transfected HeLa cells treated with vehicle or R5020, wt and S79/81A PRs behaved similarly (Fig. 3B); each receptor activated PRE-luciferase transcription to similar levels (~15- to 20-fold) in the presence of progestin (Fig. 3B). Additional characterization of the S79/81A PR mutant using confocal microscopy showed no apparent differences in subcellular localization of S79/81A PR relative to wt PR, in both the presence and absence of ligand (data not shown). Single mutant receptors (S79A and S81A) behaved similarly to the double mutant (not shown).

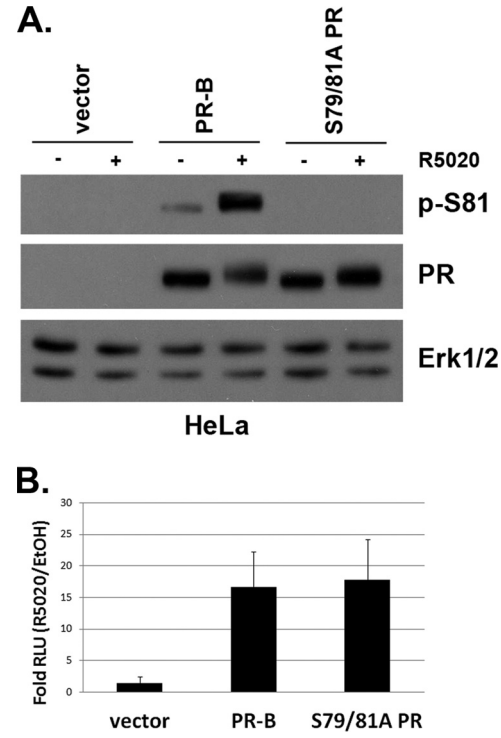

FIG. 3. S79/81A PR phospho-mutant is transcriptionally active. (A) HeLa cells were transiently transfected with wt PR-B, S79/81A PR, or empty vector. At 24 h following transfection, cells were starved for 18 h in serum-free medium and then treated with 10 nM R5020 for 60 min. Lysates were analyzed via Western blotting using p-S81, PR, and Erk1/2 antibodies. (B) HeLa cells were transiently transfected with plasmids expressing wt PR-B, S79/81A PR, or vector only, as well as a firefly PRE-luciferase reporter construct and *Renilla* expression control. At 24 h following transfection, cells were starved for 18 h in serum-free medium, followed by an 18-h 10 nM R5020 (or vehicle) treatment. Fold relative luciferase units (RLU; PRE-luciferase over *Renilla* luciferase controls) of R5020-treated cells over EtOH-treated cells is plotted. Error bars represent means ± standard deviations (SD) of results from three independent experiments.

We then created multiple clones of stable T47D-Y cell lines expressing S79/81A mutant PR (T47D-S79/81A). Cells expressing wt PR (T47D-YB) in the same parental cell line background served as controls. Western blotting demonstrated that S79/81A PR-B is expressed at similar levels relative to wt PR-B in this model system (Fig. 4A). Again, upon progestin treatment, we detected robust Ser81 phosphorylation in wt, but not S79/81A, PR-B-expressing cells. Additionally, ligand-dependent receptor downregulation, which has been shown to be augmented by MAPK-dependent PR phosphorylation (i.e., at Ser294) (58), followed a similar time course in cell lines expressing either wt or phospho-mutant S79/81A PR. To verify that ck2 expression levels remained equal among the clonal

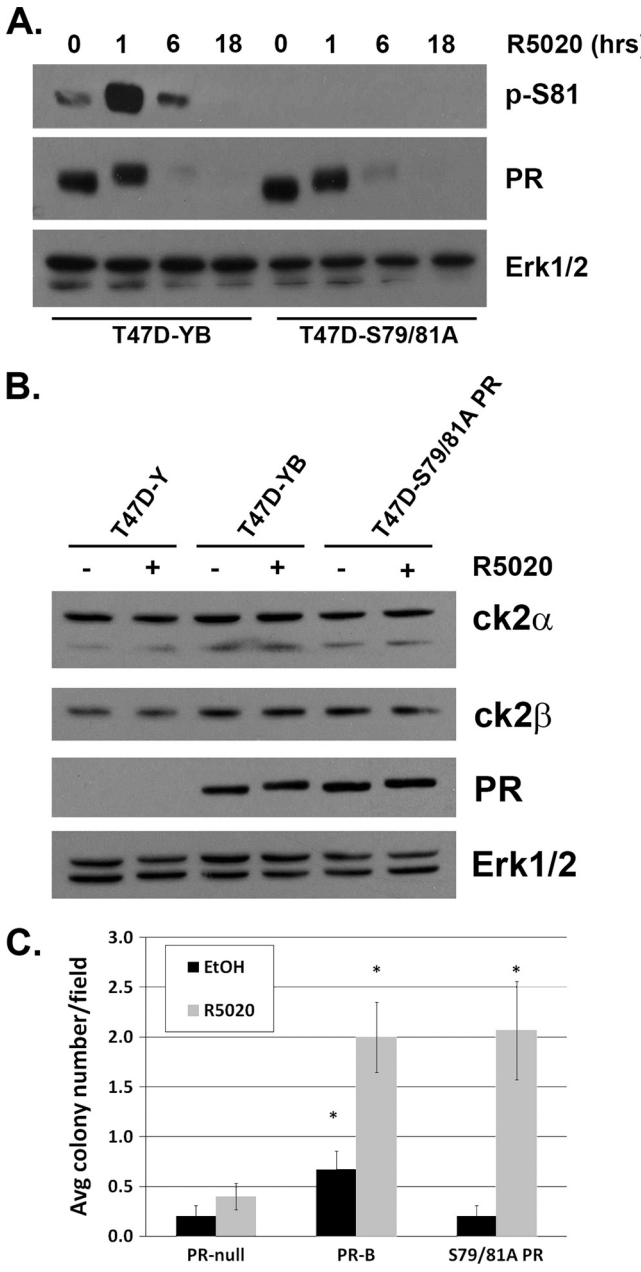

FIG. 4. Stable S79/81A PR cell lines have impaired anchorage-independent survival in soft agar. (A) T47D-Y cells stably expressing wt PR-B (T47D-YB) or S79/81A PR (T47D-S79/81A) were serum starved for 18 h and then treated with 10 nM R5020 for 0 to 18 h or vehicle (EtOH; 18 h). Lysates were analyzed by Western blotting using p-S81, PR, and Erk1/2 antibodies. (B) T47D-Y cells stably expressing wt PR-B (T47D-YB) or S79/81A PR (T47D-S79/81A) or unmodified were serum starved for 18 h and then treated with 10 nM R5020 or EtOH for 60 min. Lysates were analyzed via Western blotting using antibodies against ck2α, ck2β, PR, and Erk1/2 (loading control). (C) T47D-Y cells (PR-null) or T47D cells stably expressing PR-B or S79/81A PR were plated in soft agar containing 5% DCC medium and either EtOH or 10 nM R5020 for 21 days. Colonies were counted in 15 fields/treatment group, and error bars represent the standard errors of the means (SEM) of these measurements. Soft agar assays were performed in triplicate with similar results. Asterisks indicate statistical significance ($P < 0.05$; determined using an unpaired Student's t test) compared to the respective treatment group (EtOH or R5020) in control cells (PR-null).

cell lines, we analyzed ck2α and β protein levels via Western blotting (Fig. 4B). T47D-Y cells stably expressing wt PR-B, mutant S79/81A PR, or PR-null exhibited equal levels of both ck2 subunits; neither subunit appeared to be affected by treatment with R5020.

In soft agar assays performed *in vitro*, the proliferative and survival effects of progestins are mediated by PR-B but not PR-A (25). We therefore assayed the ability of S79/81A mutant PR to induce breast cancer cell growth in anchorage-independent soft agar assays. Stable T47D cell lines expressing either wt PR or S79/81A PR-B or PR-null were plated for soft agar colony formation assays in the presence of either vehicle or R5020 (10 nM). Following 21 days, established colonies were counted. Cells stably expressing S79/81A PR retained their ability to form colonies in response to R5020; total numbers of R5020-induced colonies were similar between cells expressing wt or S79/81A PR by the end of the 21-day assay, while PR-null cells failed to grow well in either condition (Fig. 4C; data from additional clones are available on request). Interestingly, however, cells expressing S79/81A PR formed significantly fewer colonies in the ligand-independent condition than cells expressing wt PR-B; S79/81A PR cells resembled PR-null cells in this regard (Fig. 4C). These data suggest that in the absence of exogenously added progestin, phospho-Ser81 PR may regulate genes that primarily contribute to cell survival and/or proliferation. Ligand binding is able to overcome this deficit, perhaps because the same set of genes are also highly responsive to hormone (addressed below).

Although our PRE-luciferase reporter gene analysis (Fig. 3B) indicated that S79/81A PR behaved similarly to wt PR, transcriptional activity on endogenous PR target genes offers a more sensitive and relevant readout of PR genomic action (i.e., PR-dependent regulation of complex promoters/distant enhancer elements arrayed in chromatin). Additionally, we have shown that PR phosphorylation by rapidly activated cytoplasmic protein kinases provides a mechanism for altered PR target gene selectivity, recruiting differentially phosphorylated PR species to specific gene subsets (reviewed in reference 18). Using our stable T47D cell line models, we surveyed mRNA expression of known PR target genes in the absence and presence of progestin (R5020; 0 to 18 h) by quantitative real-time PCR (qPCR). While many progestin-regulated genes were similarly expressed in cells containing either wt PR or S79/81A PR-B, others were differentially regulated (see below, Fig. 5; data from additional clones are available on request). These included the previously identified progestin-regulated genes BIRC3 (64), HSD11β2 (2), and HbEGF (4, 20, 81).

Notably, in the absence of progestin, BIRC3 (baculovirus inhibitor of apoptosis repeat 3), an antiapoptosis gene recently identified as a PR target gene (64), exhibited decreased levels of basal transcription in cells stably expressing S79/81A mutant PR relative to cells stably expressing wt PR-B (Fig. 5A, top). Unliganded PR appears to contribute to basal BIRC3 expression, as PR-null cells (T47D-Y) also contain lower levels of BIRC3 mRNA relative to cells expressing wt PR-B (T47D-YB). Thus, mutation of the Ser81 phosphorylation site in PR appears to have abrogated basal expression of this gene. Additionally, although mutant S79/81A PR was able to weakly induce BIRC3 mRNA in response to ligand, levels of this transcript never reached those observed in R5020-treated cells

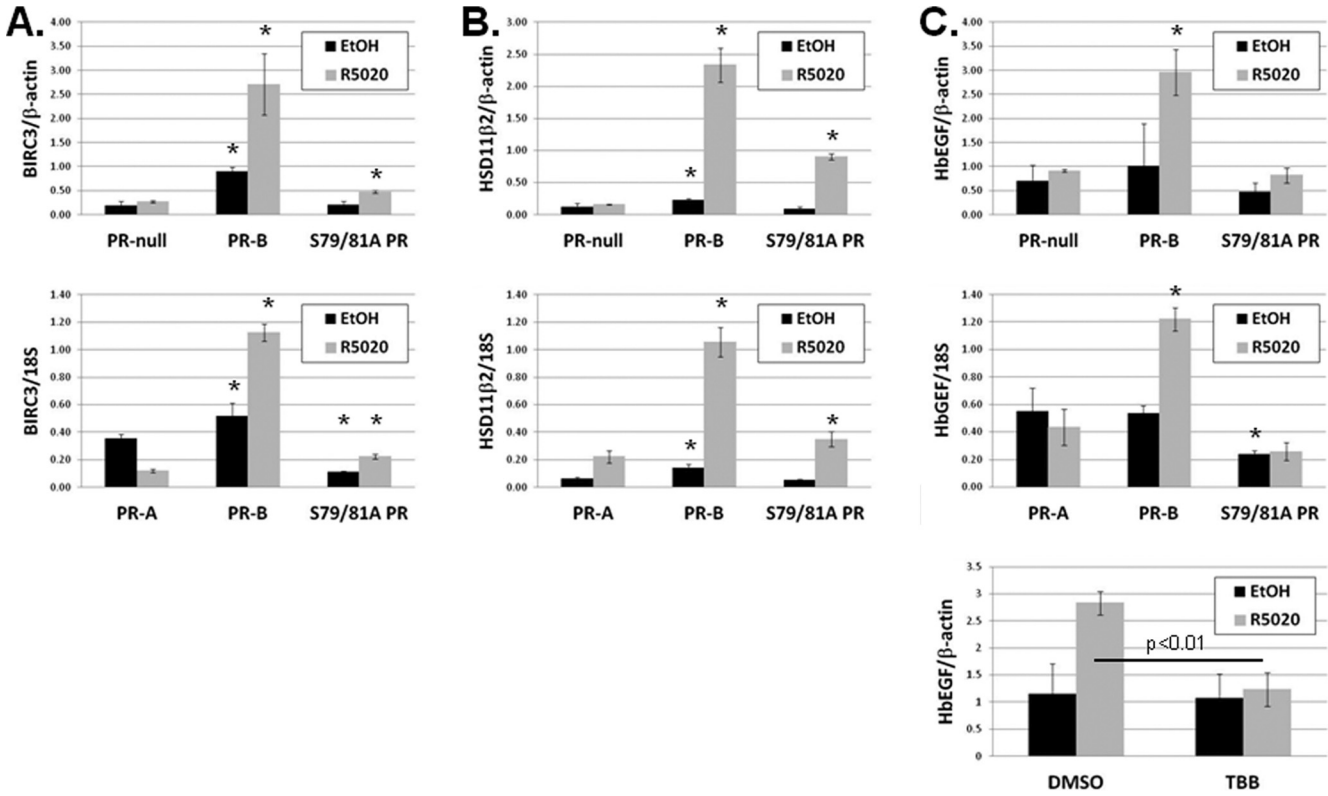

FIG. 5. Endogenous PR target gene expression is attenuated in cells containing S79/81A PR relative to wt PR. (A, B, and C) Top: T47D-Y cells stably expressing either wt PR-B or S79/81A PR, or unmodified (PR-null) cells, were starved for 18 h in serum-free medium, followed by treatment with 10 nM R5020 or EtOH for 6 h. BIRC3 (A), HSD11β2 (B), HbEGF (C), or β-actin (internal control) mRNA levels were analyzed by qPCR. Middle: T47D-Y cells stably expressing wt PR-A, PR-B, or S79/81A PR were serum starved for 18 h, followed by treatment with 10 nM R5020 or EtOH for 6 h. BIRC3 (A), HSD11β2 (B), HbEGF (C), or 18S (internal control) mRNA levels were analyzed by qPCR. Asterisks indicate statistical significance ($P < 0.05$; determined using an unpaired Student's t test) compared to the respective treatment group (EtOH or R5020) in control cells (PR-null or PR-A). Bottom (C): T47D-YB cells were starved for 18 h in serum-free medium. Cells were then pretreated with TBB (10 μM) or DMSO (vehicle) for 30 min, followed by 60 min of 10 nM R5020. HbEGF and β-actin (internal control) mRNA expression was analyzed using qPCR. Error bars represent means ± SD of triplicate measurements.

containing wt PR-B. Finally, T47D cells stably expressing PR-A (T47D-YA), and thus lacking the BUS region containing Ser81, displayed significantly lower basal expression of BIRC3 and failed to respond to progestin relative to cells expressing wt PR-B (Fig. 5A, bottom), indicating that the structural requirements for regulation of this gene (basal and ligand dependent) are localized to the segment of PR unique to the B isoform, which includes the Ser81 phosphorylation site. Together, these data indicate that phosphorylation at PR-B Ser81 significantly contributes to the basal expression of BIRC3 and is also required for robust responses to ligand.

HSD11β2 (11β-hydroxysteroid dehydrogenase type 2), a dehydrogenase enzyme that mediates tissue-specific metabolism of glucocorticoids (9), has previously been identified as a cancer-associated proliferative protein (40) and a progestin-responsive gene (2, 21). HSD11β2 behaved similarly to BIRC3 in that basal mRNA levels were significantly decreased in cells containing mutant S79/81A PR, as well as in PR-null cells, relative to wt PR-B-expressing cells, again strongly suggesting that wt PR Ser81 phosphorylation is responsible for the maintenance of basal transcription of this gene (Fig. 5B, top). Similar to the regulation of BIRC3, cells containing S79/81A PR further enhanced HSD11β2 mRNA expression in response to ligand, while overall transcript levels remained significantly lower relative to those induced in cells expressing wt PR-B. Finally, cells stably expressing PR-A contained HSD11β2 mRNA levels similar to those seen in S79/81A PR cells (both basally and in response to ligand), again suggesting that regulation of this gene is linked to PR-B-specific phosphorylation of Ser81 (Fig. 5B, bottom). These data indicate that PR-B Ser81 phosphorylation primarily regulates the basal expression of these genes (BIRC3, HSD11β2) but can also alter the magnitude of their response to hormone. Taken together with the above effects on soft agar colony formation (Fig. 4C), our data suggest that phospho-Ser81 PR contributes to gene regulation and breast cancer cell survival, even when progestins are absent or limiting.

HbEGF (heparin-binding epidermal growth factor-like growth factor) is a well-characterized phosphorylation-sensitive PR target gene shown to be important for the growth of mammary epithelial cells (4, 16, 81). In cells expressing wt PR-B, HbEGF mRNA levels were responsive to ligand (Fig. 5C, top). In contrast, cells expressing mutant S79/81A PR failed to induce HbEGF mRNA in response to R5020. Interestingly, in contrast to the previously discussed genes (Fig. 5A and B), basal HbEGF transcript levels remained comparable in

the absence of ligand in cells expressing either wt PR-A or PR-B, mutant S79/81A PR, or no PR, suggesting that PR does not influence basal transcription of this gene. Cells expressing PR-A and treated with progestin failed to induce HbEGF, again implicating the Ser81-containing region unique to PR-B in the progestin-dependent regulation of this gene (Fig. 5C, middle). Finally, cells treated with the ck2 inhibitor, TBB, also failed to induce HbEGF mRNA in response to ligand (Fig. 5C, bottom). Together, these data implicate the kinase activity of ck2, presumably through direct phosphorylation of PR Ser81, in progestin-induced upregulation of HbEGF mRNA expression.

To verify that the transcriptional differences described above (BIRC3, HSD11β2, and HbEGF) between cells expressing wt PR and S79/81A PR indeed reflect a functional requirement for phosphorylation of PR Ser81 in gene activation, rather than altered kinetics of gene activation, we analyzed mRNA isolated from cells following a time course of R5020 treatment (0 to 18 h) (Fig. 6). Impaired transcription observed in S79/81A PR-B-expressing cells relative to cells containing wt PR-B remained significant throughout this time course. Absolute mRNA levels (HbEGF and HSD11β2) became equal only after the peak of transcriptional activation, when mRNA levels began to decline. These data support the conclusion that PR Ser81 is required for absolute regulation of selected PR target genes over an extended time course.

Notably, the expression of well-characterized PR target genes, including c-Fos, tissue factor (TF), and EGFR (epidermal growth factor receptor) (38, 55, 56) was not differentially affected either basally or in response to ligand in cells expressing mutant S79/81A PR compared to expression of those expressing wt PR (data not shown). These genes represent a diverse spectrum of progestin-responsive promoters that display a variety of transcriptional kinetics (i.e., peak mRNA expression) following ligand treatment at 1 h (c-Fos), 6 h (TF), and 18 h (EGFR). These data suggest that mutation of the Ser81 phosphorylation site has not disrupted the ability of PR to activate endogenous target genes via general mechanisms (i.e., that may alter all PR transcriptional complexes or effect PR localization), indicating that the genes discussed above are uniquely regulated by phospho-PR Ser81. Results repeated in multiple clones of T47D cells stably expressing wt and phospho-mutant PRs (data available on request).

There are few reports of ligand-independent PR action. Surprisingly, both BIRC3 and HSD11β2 exhibited basal upregulation in cells expressing wt but not phospho-mutant PR-B (Fig. 5A and B and data available on request). To confirm that these genes are regulated by phospho-PRs independently of progestin, we employed an isogenic model of inducible PR expression. T47D-iEV (empty vector) and T47D-iPR-B (inducible wt PR-B) cells were treated with a small molecule inducer (AP21967; AP) or vehicle (EtOH) for 48 h; Western blotting confirmed PR-B expression (Fig. 7A, inset). In the absence of progestin, mRNA isolated from these cells showed significant increases in both BIRC3 (Fig. 7A, left) and HSD11β2 (Fig. 7A, right) transcripts only when PR-B was expressed. In contrast, transcription of two control genes, HbEGF, a ligand-dependent PR Ser81-regulated gene that is not basally regulated by wt PR (Fig. 5C), and TF, a gene that is not responsive to PR Ser81 phosphorylation, were not significantly affected by PR expression (data not shown). These data confirm that basal transcription of these phospho-Ser81-regulated genes is indeed PR dependent, but independent of exogenously added progestins.

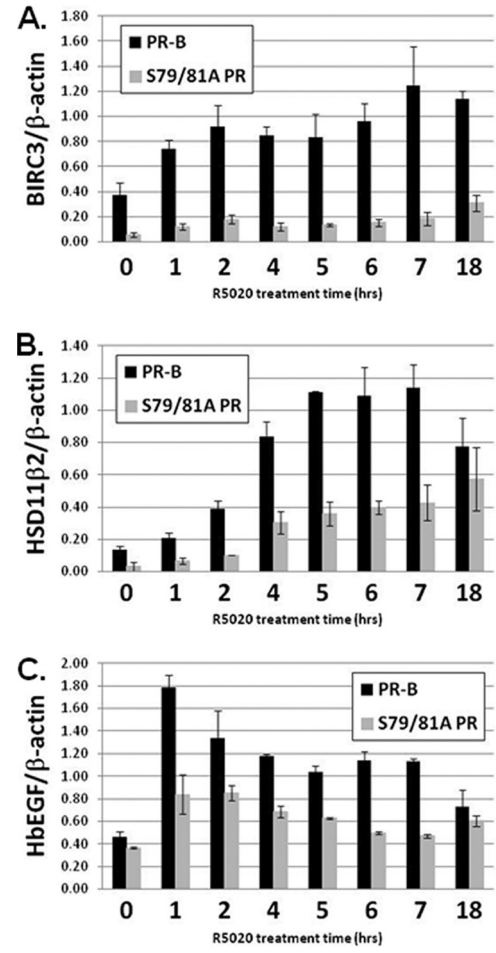

FIG. 6. Time course of endogenous gene expression in wt and S79/81A PR-expressing cells. T47D-Y cells stably expressing either wt PR-B or S79/81A PR were starved for 18 h in serum-free medium, followed by treatment with 10 nM R5020 for 0 to 18 h. BIRC3 (A), HSD11β2 (B), HbEGF (C), or β-actin (internal control) mRNA levels were analyzed by qPCR. Statistical significance ($P < 0.05$; determined using an unpaired Student's t test) was achieved for all time points when comparing wt PR-B- and S79/81A PR-expressing cells with the following exceptions: HSD11β2 (18 h) and HbEGF (0 and 18 h). Error bars represent means ± SD of triplicate measurements.

Ligand-independent regulation of selected PR target genes provides a mechanism for PR coupling to cell cycle regulation in rapidly dividing cells. To link ck2-induced (ligand-independent) PR Ser81 phosphorylation (occurring in G_1/S phase; Fig. 2D) to functional changes in gene expression, we examined BIRC3 mRNA levels during the G_1/S phase of the cell cycle in synchronized populations of T47D cells either lacking PR or stably expressing wt or S79/81A PR-B. Upon G_1/S phase synchronization of PR-null cells, we observed PR-independent (G_1/S-dependent) increased BIRC3 mRNA expression (Fig. 7B, left). However, cells containing wt PR-B, but not phospho-mutant S79/81A PR, exhibited a further significant increase in BIRC3 mRNA levels (relative to PR-null cells). TF mRNA

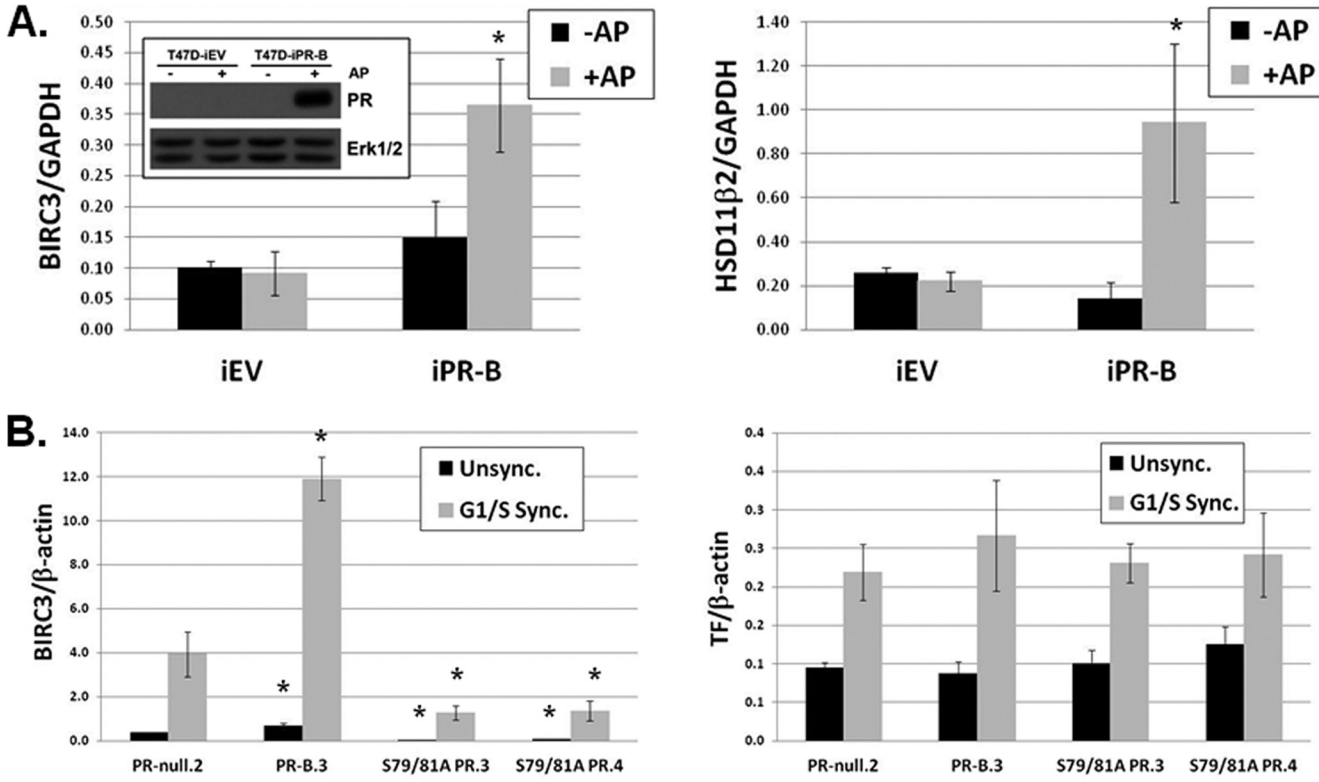

FIG. 7. Basal transcriptional regulation of phospho-Ser81-dependent genes. (A) T47D-iEV and T47D-iPR-B cells were treated for 48 h with 1 nM AP21967 (+AP and −AP) or vehicle (EtOH) to induce PR-B expression (inset). BIRC3 (left), HSD11β2 (right), or GAPDH (internal control) mRNA levels were analyzed by qPCR. Asterisks (*) indicate statistical significance ($P < 0.05$; determined using an unpaired Student's *t* test) compared to the respective treatment group (+AP or −AP) in control cells (iEV), as well as in response to treatment (+AP or −AP) within each cell line. (B) T47D-Y cells stably expressing wt PR-B (PR-B.3), S79/81A PR (S79/81A PR.3 and S79/81A PR.4), or PR-null (PR-null.2) were G_1/S synchronized as described in the legend to Fig. 2D. BIRC3 (left), TF (right), or β-actin (internal control) mRNA levels were analyzed by qPCR. Asterisks indicate statistical significance ($P < 0.05$; determined using an unpaired Student's *t* test) compared to the respective treatment group (Unsync. or Sync.) in control cells (PR-null.2). Error bars represent means ± SD of triplicate measurements.

levels were similar among G_1/S-synchronized cells, independent of their PR status (Fig. 7B, right). These data indicate that BIRC3, a gene regulated basally in response to Ser81 PR phosphorylation, is transcriptionally activated during G_1/S phase, a period when ck2-dependent PR Ser81 phosphorylation occurs in the absence of progestins.

Recruitment of phospho-Ser81 PR and ck2 to target gene promoters. To confirm direct regulation of PR target genes by phospho-Ser81 PR-B, we performed chromatin immunoprecipitation (ChIP) assays. *In silico* analysis of promoter and enhancer regions of the BIRC3 gene revealed several putative full-length PRE binding sites, including sites located just after the transcriptional start site (Fig. 8A). ChIP analysis was performed on lysates from EtOH- or R5020-treated cells stably expressing wt or S79/81A PR, or from PR-null cells, using PR-specific antibodies. In the presence of ligand, we detected robust recruitment (~70-fold) of wt PR to a full-length PRE (PRE1) located within 4 kb (downstream) of the BIRC3 transcriptional start site (Fig. 8B). This is in contrast to much-decreased S79/81A PR recruitment (~10-fold) to the same area observed in side-by-side assays performed from R5020-treated cells (Fig. 8B). PR-B recruitment to PRE1 appeared to be highly specific, as other areas tested within the proximal and distal promoter regions were negative for PR binding (data not shown). In the presence of progestin, wt and S79/81A PR-B were equally recruited to a PRE located in the TF promoter region (data available on request), a gene shown earlier not to be regulated by Ser81 phosphorylation. These data indicate that decreased recruitment of S79/81A PR to the PRE1-containing region of BIRC3 is specific to this phospho-Ser81-responsive gene and does not represent a general defect in DNA-binding and/or tethering to general transcription factors by mutant S79/81A PR. Interestingly, although we observed significant differences in the basal levels of BIRC3 mRNA expression between cells containing wt and S79/81A PR (Fig. 5 and 6), we did not detect appreciable recruitment of PR to PRE1 in the absence of progestin. It is possible that PRE1 primarily regulates the ligand-activated transcriptional response of this gene, whereas another PRE(s) in the region may regulate basal activities and would, therefore, not be detected in our ChIP analyses (focused on PRE1).

To determine if ck2, the kinase responsible for PR Ser81 phosphorylation and, therefore, functional activation of PR-B at Ser81-dependent target genes, was also present at this site, we repeated our ChIP assays using antibodies directed against ck2α, one of the active subunits comprising the ck2 holoenzyme. Interestingly, ck2α was also strongly recruited to PRE1 in cells containing wt PR-B (~8-fold), but not in those con-

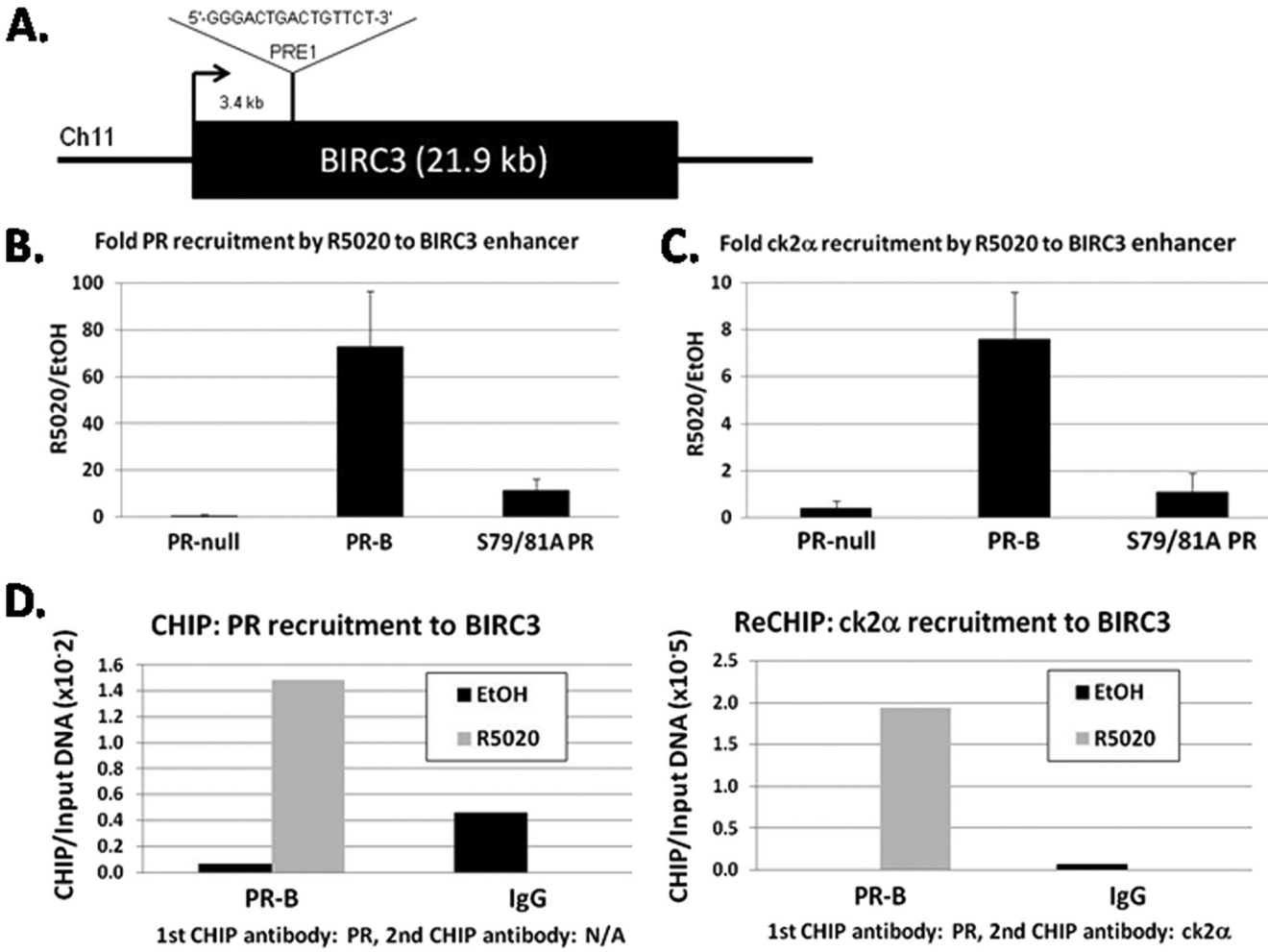

FIG. 8. Decreased recruitment of S79/81A PR and ck2α to a PRE-containing BIRC3 enhancer region. (A) Schematic of PRE1 location in BIRC3 gene. PRE1 is located 3.4 kb downstream of the transcriptional start site (denoted with the arrow). The sequence of PRE1 is shown. (B and C) T47D-Y cells stably expressing either wt PR-B or S79/81A PR or unmodified cells (PR-null) were serum starved for 18 h. Cells were then treated with EtOH or 10 nM R5020 for 60 min. Fixed lysates were subjected to ChIP with antibodies against PR-B (B) or ck2α (C), and qPCR was performed on the isolated DNA using primers designed to amplify PRE1. Fold recruitment of PR or ck2α in R5020 conditions over EtOH is shown. Error bars represent means ± SEM of results from triplicate experiments. (D) T47D-Y cells stably expressing wt PR-B were serum starved for 18 h. Cells were then treated with EtOH or 10 nM R5020 for 60 min. Fixed lysates were subjected to ChIP with antibodies against PR-B (left), followed by ck2α (right), and qPCR was performed on the isolated DNA using primers designed to amplify PRE1 in BIRC3. Species-specific IgG antibodies were used as controls (IgG). ChIP-reChIP experiments were performed in duplicate, and a representative experiment is shown.

taining S79/81A PR (~1-fold) (Fig. 8C). These data indicate that in the presence of progestin, both wt PR-B and its activating kinase, ck2, are recruited to PR-binding sites within transcriptional regulatory regions of BIRC3. Moreover, surprisingly, mutation of PR Ser81 greatly diminished not only PR-B recruitment to this PRE but recruitment of ck2 as well, suggesting that phosphorylation of this residue is important for the formation of stable protein complexes that are associated with direct regulation of this gene.

To determine if PR and ck2α were corecruited to this site in the BIRC3 enhancer, we performed ChIP-reChIP analysis (Fig. 8D). In cells expressing wt PR-B, sequential immunoprecipitations using PR antibodies (Fig. 8D, left) followed by ck2α antibodies (Fig. 8D, right) showed that the two proteins were present together at PRE1. This interaction was detected only in cells following treatment with R5020. Reversing the order of the antibodies for the ChIP-reChIP experiment yielded similar results (data not shown). We conclude that phospho-Ser81 PR-B provides a platform for the early recruitment of ck2-containing transcriptional complexes that direct promoter-specific PR target gene regulation.

DISCUSSION

Our studies reveal novel hormone and cell cycle-dependent regulation of PR Ser81 by ck2, a protein kinase tightly associated with prosurvival and uncontrolled proliferative phenotypes that characterize human malignancy. We show that progestin induces robust ck2-dependent phosphorylation of PR Ser81. Interestingly, this ck2-dependent event also occurs in the absence of added PR ligands, during the G_1/S transition point of the cell cycle (Fig. 2). This result highlights the im-

portant linkage that exists between PR and cell cycle regulation (22). Notably, hormone-dependent PR Ser81 phosphorylation is a relatively rapid event, occurring as early as 10 min following treatment with PR ligands (R5020, RU486; Fig. 1). Other potent mitogenic stimuli, including EGF and serum, failed to appreciably induce phosphorylation at this site (data available on request). Protein kinase inhibitor studies confirmed that ck2 is the kinase primarily responsible for PR Ser81 phosphorylation *in vivo* (Fig. 2). Mutational analysis revealed that phospho-mutant S79/81A PR, while equally transcriptionally active as wt PR in PRE-luciferase reporter gene assays (i.e., a minimal artificial promoter), exhibited dramatically impaired recruitment and transcriptional responses relative to wt PR on selected endogenous PR target genes (Fig. 5 to 8). PR Ser81 phosphorylation is required for efficient PR and ck2 recruitment to PRE1, located within the BIRC3 downstream enhancer region (Fig. 8). Taken together, these data indicate that PR/ck2 complexes may regulate a distinct subset of phospho-Ser81-specific PR-B target genes in both the presence and the absence of ligand (i.e., in proliferating/cycling cells). Our findings provide novel insight into how PR-B may contribute to breast cancer prosurvival and tumor progression, even when hormone concentrations are limiting.

Role of PR phosphorylation events in breast cancer models. Phosphorylation can impact diverse properties of the respective substrate. Direct phosphorylation of PR at specific amino-terminal Ser residues has been shown to alter receptor stability, localization, protein complex formation, dimerization, transcriptional activity, and promoter selectivity (18, 75). Data presented here indicate that tightly regulated (i.e., in response to hormone-binding and/or during G_1/S transition) Ser81 phosphorylation directs target gene specificity; we identified at least three PR target genes that are differentially regulated by phosphorylation at this site. One class of genes is altered in both the presence and the absence of progestin (BIRC3 and HSD11β2), while HbEGF is an example of a gene whose expression is primarily ligand and ck2 dependent (i.e., induced via hormone-regulated PR Ser81 phosphorylation), lacking regulation in the absence of ligand. The precise mechanism(s) through which Ser81 phosphorylation alters PR-B target gene specificity is not clear, but such phosphorylation might occur via complex mechanisms that may include altered formation of transcriptional complexes and/or recognition/binding affinity for PRE elements and associated regulatory elements, thus altering early events in promoter recruitment (Fig. 8 and further discussed below).

Related to this finding, phosphorylation on Ser81 contributes in part to PR isoform specificity (Fig. 5). The two predominant PR isoforms, PR-B and PR-A, have overlapping but distinct transcriptional profiles (64) and have tissue-specific effects on growth (54), presumably through activation of different subsets of target genes. These receptors are generally expressed at a 1:1 ratio (i.e., equal levels) in normal mammary epithelial cells, but the ratio of expression is often altered in breast cancers (53). The full-length receptor, PR-B, contains an N-terminal region (the BUS) unique to PR-B where Ser81 is located. Data presented here showing that PR-B-activated gene transcription is lost on selected genes following mutation of the Ser81 phosphorylation site, and that mutant S79/81A PR-B mimics PR-A in this regard, suggest that Ser81 may be critical for PR-B versus PR-A target gene specificity. Related to this concept, we have begun to explore the possibilities of altered PR-A/B protein-protein interactions with associated transcriptional coactivators, corepressors, and other cofactors. Changes in further posttranslational modifications of PR (sumoylation, acetylation, ubiquitination, subsequent multisite phosphorylation events) may also be isoform specific and dictated in part by early phosphorylation events (16) and/or sequential events (15) but are outside the scope of the present study.

Transcriptional mechanisms are highly ordered and dynamic processes, characterized by waves of interactions between DNA and dozens of regulatory molecules. Given this enormous complexity, the precise role of ck2-dependent PR Ser81 phosphorylation may remain elusive. Notably, preliminary cell fractionation and confocal experiments suggested identical subcellular localization of wt PR and S79/81A PR, independent of ligand (data not shown). Additionally, the rate of ligand-dependent downregulation/receptor turnover appeared to be unaltered by Ser79/81 mutation (Fig. 4). Effects on PR dimerization are unlikely, as S79/81A PR was able to activate PRE-luciferase transcription (Fig. 3) as well as regulate other endogenous PR target genes to levels equal to that of wt PR (c-Fos, TF, EGFR). These data indicate that mutant S79/81A PR is a fully functional transcription factor for some promoters but not others (i.e., promoter selectivity is primarily altered). Interestingly, much less phospho-mutant PR protein appeared to be recruited to a PRE located in the BIRC3 enhancer region relative to wt PR-B (Fig. 8), while recruitment to other Ser79/81-independent genes (TF; data available on request and Garabedian) was unaffected. This finding suggests a block at some early event required for efficient PR/DNA recognition and/or interaction. Recent work from Blind and Garabedian. (6) also suggests that phospho-specific steroid receptor isoforms are differentially recruited to the promoters of specific genes based on their phosphorylation status. Using ChIP analysis, the authors showed that phosphorylation patterns on the glucocorticoid receptor (GR) dictate which gene promoters those phospho-GRs were recruited to, the kinetics of that respective recruitment, and, therefore, which GR target genes were subsequently activated (6). Our data showing decreased recruitment of mutant S79/81A PR to select PR target genes (Fig. 8) are in concordance with this finding and suggest that this mechanism of transcriptional regulation may be a characteristic shared by many steroid receptors.

In addition to PR recruitment to the BIRC3 enhancer region, data presented here also show that ck2α, the kinase responsible for Ser81 phosphorylation of PR, is similarly recruited to the same region in the presence of progestin (Fig. 8). ChIP-reChIP experiments demonstrated that wt PR and ck2α reside together in the same DNA-bound protein complexes. Surprisingly, less ck2α is recruited to the BIRC3 enhancer region in cells expressing mutant S79/81A PR. These data suggest that PR Ser81 phosphorylation mediates the formation of stable transcriptional complexes that may contain multiple proteins/phospho-proteins. Other factors (not assayed herein), functioning similarly to estrogen receptor (ER) or AR-associated pioneer factors (45), may require ck2-dependent PR Ser81 phosphorylation for assembly and/or stable association (i.e., that can be detected upon cross-linking); no obvious se-

quences that could serve as binding sites for additional PR- or ck2-associated factors were noted in the BIRC3 or HSD11β2 gene regulatory regions. Notably, Narayanan et al. (57) showed that cyclin A and PR are recruited to PRE regions within the MMTV promoter (stably incorporated into the T47D cell genome). In these studies, the interaction between cyclin A and active cdk2 was necessary to stimulate PR transcriptional activity, primarily via phosphorylation of SRC-1 coactivator molecules (57). These findings using an exogenous MMTV promoter system, and our data presented herein, performed on endogenous PR target genes expressed in breast cancer cells, suggest that phosphorylation events and subsequent transcriptional activation of PR are tightly linked at selected promoters and that the protein kinases responsible for these modifications (of PR and/or coregulators) are an integral part of PR-containing transcriptional complexes. ER was recently shown to associate with ERK2 and CREB at selected estrogen-responsive genes important for breast cancer cell proliferation, although the required substrate(s) in transcriptional complexes that are phosphorylated by ERK2 activity (i.e., possibly CREB) has yet to be defined (48).

Notably, weak PR Ser81 phosphorylation occurred in the absence of progestins (Fig. 1 to 4). However, this site was potently phosphorylated in cells entering the G_1/S boundary (Fig. 2D), as in response to progestin. Ligand binding to PR sets up an exquisite program of cell cycle synchronization wherein cells enter S phase following precisely timed regulation of cell cycle mediators (reviewed in reference 22). Indeed, PR target genes include cyclins (D, E, and A) and cdk inhibitors (p21 and p27), and progestin-treated breast cancer cells are known to pause or accumulate at the G_1/S boundary (30). Given the tight coupling of PR to cell cycle control, it is perhaps not surprising that selected PR target genes depend upon PR Ser81 phosphorylation for regulation both in the presence (HbEGF) and absence (BIRC3 or HSD11β2) of ligand. Ligand-independent PR gene regulation may provide important clues to how ck2 is regulated during cell cycle traverse. Protein complex formation involving Ser81-phosphorylated PR and ck2 is the topic of future studies.

Functional significance of ck2 and PR Ser81 target gene regulation in breast cancer. The Ser/Thr protein kinase ck2 is upregulated in every cancer studied thus far (72). Although ck2 itself does not appear to be an oncogene, it is thought that ck2 works in an oncogenic fashion by potentiating the activity of other oncogenes and progrowth signaling molecules that function as its major substrates (reviewed in reference 74). For example, numerous studies have shown that ck2 overexpression promotes tumorigenesis in existing transgenic mouse models of cancer (11, 39, 41, 42). In the context of breast cancer, where progestins have been implicated as a risk factor for tumor development and early progression (1, 5, 12), overexpressed ck2 could further enhance the oncogenic potential of PR through inappropriate phosphorylation (on Ser81). Notably, the genes that are transcriptionally regulated by PR Ser81 phosphorylation have been shown to be important in cell growth and have each been identified in various types of cancer, including breast cancer. BIRC3 is an anti apoptosis protein belonging to the inhibitor of apoptosis (IAP) family of proteins (65). IAPs bind to and inhibit other pro-death-associated proteins, such as caspases, thereby preventing apoptosis (44). BIRC3, a mammalian-specific IAP also known as cellular IAP2 (cIAP2), is overexpressed, along with other closely related IAP family members, in breast cancer (28). HSD11β2 is a dehydrogenase enzyme that is responsible for the tissue-specific metabolism of glucocorticoids (reviewed in reference 9). Specifically, HSD11β2 expression has proliferative effects, especially in tumors, through inactivation of the anti proliferative effects of GR (36). Of note, HSD11β2 is upregulated in many different cancers, including breast, whereas the corresponding normal nonneoplastic tissue normally lacks HSD11β2 expression (36, 40). As a PR target gene, HSD11β2 may be an important mediator of progestin action. Finally, HbEGF, a gene shown here to be regulated by ligand-induced PR Ser81 phosphorylation, has been shown to contribute to mammary cell proliferation and breast cancer cell growth (4, 20). Moreover, ck2 is frequently upregulated in breast cancer. This fact, coupled with our findings that phospho-Ser81 PR can drive the expression of genes that clearly contribute to breast cancer biology, suggests a scenario for ck2-high breast tumors, in which PR may be inappropriately or persistently phosphorylated on Ser81 (i.e., either basally or in response to ligand) and thereby contribute to a hyperproliferative state. Indeed, we observed increased ligand-independent soft agar colony formation in cells expressing wt PR-B relative to cells expressing S79/81A PR and PR-null cells. Thus, the basal level of anchorage-independent growth was abrogated in cells expressing phospho-mutant S79/81A PR (Fig. 4C); cells expressing PR-A also fail to grow in soft agar (25). Related to this finding, we suspect that many additional prosurvival and/or proliferative genes are regulated by phospho-Ser81 PR. The identification of a more complete Ser81-regulated gene signature in breast cancer cells awaits detailed gene array analyses. Additionally, the presence of phospho-PR Ser81 in breast tumors may provide a marker of activated PRs in S-phase cells (in progress).

Due to the diverse nature and subcellular distribution of the >300 substrates of ck2, it is not surprising that ck2 has been localized to nearly every cellular compartment, including, but not limited to, the nucleus, cytoplasm, plasma membrane, and mitochondria (reviewed in reference 26). Conflicting reports exist regarding a correlation between ck2 localization and cell cycle; this discrepancy is likely due to cell type-specific differences in ck2 distribution. Reports indicate that ck2 localization (either the holoenzyme or specific subunits) shifts to predominantly nuclear during the G_1 phase of the cell cycle and at the G_1/S border (51, 78); we have also detected a similar shift in PR localization in G_1/S synchronized cells (data not shown). Phosphorylation of PR Ser81 in the absence of ligand (observed in cells arrested at the G_1/S transition; Fig. 2D) may be regulated as a consequence of increased nuclear accumulation of ck2 and PR observed at this stage of the cell cycle. In addition, work from the Ahmed lab (reviewed in reference 32) showed that in response to androgenic or growth factor signals in prostate cancer cells, ck2 localization was strongly nuclear and specifically associated with the nuclear matrix and chromatin, areas of high transcriptional activity (33). Progestins may work similarly to their androgenic counterparts and direct PR to the ck2-containing nuclear compartment, subsequently inducing prolonged phosphorylation of PR Ser81. Interest-

ingly, PR nuclear entry appears to precede Ser81 phosphorylation (data not shown), similar to the pattern recently described for PR phosphorylation on Ser294 and Ser400 (17).

Significantly, nearly 70% of breast cancers express both ER and PR at the time of diagnosis, in contrast to PR/ER expression in just 7 to 10% of normal breast luminal epithelium (67). As steroid hormone receptor (SR)-positive tumors progress, they frequently become hormone independent while retaining receptor expression, indicating an early switch to autocrine or paracrine growth factor signaling (60). In addition, many breast cancers have upregulated protein kinases, such as MAPK, c-Src, cdk2, and ck2, which can modify and hyperactivate PR (29, 69, 72, 77). Recently, progesterone was shown to mediate mammary stem cell self-renewal via paracrine mechanisms in which secreted factors (Wnt, RANKL) derived from PR-positive cells influence the PR-null stem cell niche (37). In PR-positive breast cancer cells, PR action drives proliferation, prosurvival signaling, and early invasion primarily by autocrine mechanisms (10, 25, 61). In an environment where steroid hormones are no longer required to drive cellular proliferation (i.e., during SR-positive tumor progression), the increased expression and constitutive activation of PR-activating protein kinases may promote increased cell survival and uncontrolled growth (i.e., in the face of endocrine therapies primarily directed against ER). Understanding how mitogenic protein kinases, such as ck2, alter PR phosphorylation and function is critical to fully understanding breast tumor etiology and developing better targeted therapies. Due to the ubiquitous nature of ck2 and its prevalence in many different types of cancer, there has been much interest in the development of ck2 inhibitors as anti cancer agents (73). Clinical ck2 inhibitors, in combination with more specific anti-progestins (new classes of selective progesterone receptor modulators or SPRMs), could provide an effective combination of targeted therapy for breast cancer treatment.

ACKNOWLEDGMENTS

We thank Khalil Ahmed (University of Minnesota) for providing test aliquots of ck2 inhibitors. We thank Andrea R. Daniel (Lange lab) for helpful comments on the manuscript and Todd Knutson (Lange lab) for supplying cDNA isolated from the inducible control and PR-B isogenic cell system.

This work was supported by NIH/NCI grant number R01 CA123763 (C.A.L.), Department of Defense Post-Doctoral Fellowship number USDOD ARMY/W81XWH-09-1-0639 PK0001 (C.R.H.), and NIH Institutional Training Grant number T32 CA009138 (C.R.H. and G.E.D.).

REFERENCES

1. **Anderson, G. L., et al.** 2004. Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. JAMA **291:**1701–1712.
2. **Arcuri, F., et al.** 2000. Progestin regulation of 11beta-hydroxysteroid dehydrogenase expression in T-47D human breast cancer cells. J. Steroid Biochem. Mol. Biol. **72:**239–247.
3. **Bagowski, C. P., J. W. Myers, and J. E. Ferrell.** 2001. The classical progesterone receptor associates with p42 MAPK and is involved in PI3-K signaling in *Xenopus* oocytes. J. Biol. Chem. **276:**37708–37714.
4. **Beerli, R. R., and N. E. Hynes.** 1996. Epidermal growth factor-related peptides activate distinct subsets of ErbB receptors and differ in their biological activities. J. Biol. Chem. **271:**6071–6076.
5. **Beral, V.** 2003. Breast cancer and hormone-replacement therapy in the Million Women Study. Lancet **362:**419–427.
6. **Blind, R. D., and M. J. Garabedian.** 2008. Differential recruitment of glucocorticoid receptor phospho-isoforms to glucocorticoid-induced genes. J. Steroid Biochem. Mol. Biol. **109:**150–157.
7. **Boonyaratanakornkit, V., et al.** 2001. Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases. Mol. Cell **8:**269–280.
8. **Brisken, C., et al.** 1998. A paracrine role for the epithelial progesterone receptor in mammary gland development. Proc. Natl. Acad. Sci. U. S. A. **95:**5076–5081.
9. **Bush, I. E., S. A. Hunter, and R. A. Meigs.** 1968. Metabolism of 11-oxygenated steroids. Metabolism in vitro by preparations of liver. Biochem. J. **107:**239–258.
10. **Carvajal, A., et al.** 2005. Progesterone pre-treatment potentiates EGF pathway signaling in the breast cancer cell line ZR-75. Breast Cancer Res. Treat. **94:**171–183.
11. **Channavajhala, P., and D. C. Seldin.** 2002. Functional interaction of protein kinase CK2 and c-Myc in lymphomagenesis. Oncogene **21:**5280–5288.
12. **Chlebowski, R. T., et al.** 2003. Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: the Women's Health Initiative randomized trial. JAMA **289:**3243–3253.
13. **Chlebowski, R. T., et al.** 2009. Breast cancer after use of estrogen plus progestin in postmenopausal women. N. Engl. J. Med. **360:**573–587.
14. **Cicatiello, L., et al.** 2004. Estrogens and progesterone promote persistent CCND1 gene activation during G_1 by inducing transcriptional derepression via c-Jun/c-Fos/estrogen receptor (progesterone receptor) complex assembly to a distal regulatory element and recruitment of cyclin D1 to its own gene promoter. Mol. Cell. Biol. **24:**7260–7274.
15. **Clemm, D. L., et al.** 2000. Differential hormone-dependent phosphorylation of progesterone receptor A and B forms revealed by a phosphoserine site-specific monoclonal antibody. Mol. Endocrinol. **14:**52–65.
16. **Daniel, A. R., E. J. Faivre, and C. A. Lange.** 2007. Phosphorylation-dependent antagonism of sumoylation derepresses progesterone receptor action in breast cancer cells. Mol. Endocrinol. **21:**2890–2906.
17. **Daniel, A. R., et al.** 2010. The progesterone receptor hinge region regulates the kinetics of transcriptional responses through acetylation, phosphorylation, and nuclear retention. Mol. Endocrinol. **24:**2126–2138.
18. **Daniel, A. R., T. P. Knutson, and C. A. Lange.** 2009. Signaling inputs to progesterone receptor gene regulation and promoter selectivity. Mol. Cell Endocrinol. **308:**47–52.
19. **Daniel, A. R., and C. A. Lange.** 2009. Protein kinases mediate ligand-independent derepression of sumoylated progesterone receptors in breast cancer cells. Proc. Natl. Acad. Sci. U. S. A. **106:**14287–14292.
20. **Daniel, A. R., et al.** 2007. Linkage of progestin and epidermal growth factor signaling: phosphorylation of progesterone receptors mediates transcriptional hypersensitivity and increased ligand-independent breast cancer cell growth. Steroids **72:**188–201.
21. **Darnel, A. D., T. K. Archer, and K. Yang.** 1999. Regulation of 11beta-hydroxysteroid dehydrogenase type 2 by steroid hormones and epidermal growth factor in the Ishikawa human endometrial cell line. J. Steroid Biochem. Mol. Biol. **70:**203–210.
22. **Dressing, G. E., and C. A. Lange.** 2009. Integrated actions of progesterone receptor and cell cycle machinery regulate breast cancer cell proliferation. Steroids **74:**573–576.
23. **Duncan, J. S., et al.** 2008. An unbiased evaluation of CK2 inhibitors by chemoproteomics: characterization of inhibitor effects on CK2 and identification of novel inhibitor targets. Mol. Cell. Proteomics **7:**1077–1088.
24. **Faivre, E. J., A. R. Daniel, C. J. Hillard, and C. A. Lange.** 2008. Progesterone receptor rapid signaling mediates serine 345 phosphorylation and tethering to specificity protein 1 transcription factors. Mol. Endocrinol. **22:**823–837.
25. **Faivre, E. J., and C. A. Lange.** 2007. Progesterone receptors upregulate Wnt-1 to induce epidermal growth factor receptor transactivation and c-Src-dependent sustained activation of Erk1/2 mitogen-activated protein kinase in breast cancer cells. Mol. Cell. Biol. **27:**466–480.
26. **Faust, M., and M. Montenarh.** 2000. Subcellular localization of protein kinase CK2. A key to its function? Cell Tissue Res. **301:**329–340.
27. **Filhol, O., and C. Cochet.** 2009. Protein kinase CK2 in health and disease: cellular functions of protein kinase CK2: a dynamic affair. Cell. Mol. Life Sci. **66:**1830–1839.
28. **Foster, F. M., et al.** 2009. Targeting inhibitor of apoptosis proteins in combination with ErbB antagonists in breast cancer. Breast Cancer Res. **11:**R41.
29. **Gregory, C. W., et al.** 2004. Epidermal growth factor increases coactivation of the androgen receptor in recurrent prostate cancer. J. Biol. Chem. **279:**7119–7130.
30. **Groshong, S. D., et al.** 1997. Biphasic regulation of breast cancer cell growth by progesterone: role of the cyclin-dependent kinase inhibitors, p21 and p27(Kip1). Mol. Endocrinol. **11:**1593–1607.
31. **Guerra, B., and O. G. Issinger.** 2008. Protein kinase CK2 in human diseases. Curr. Med. Chem. **15:**1870–1886.
32. **Guo, C., A. T. Davis, S. Yu, S. Tawfic, and K. Ahmed.** 1999. Role of protein kinase CK2 in phosphorylation nucleosomal proteins in relation to transcriptional activity. Mol. Cell. Biochem. **191:**135–142.
33. **Guo, C., S. Yu, A. T. Davis, and K. Ahmed.** 1999. Nuclear matrix targeting of the protein kinase CK2 signal as a common downstream response to androgen or growth factor stimulation of prostate cancer cells. Cancer Res. **59:**1146–1151.

34. **Horwitz, K. B., Y. Koseki, and W. L. McGuire.** 1978. Estrogen control of progesterone receptor in human breast cancer: role of estradiol and antiestrogen. Endocrinology **103:**1742–1751.
35. **Horwitz, K. B., M. B. Mockus, and B. A. Lessey.** 1982. Variant T47D human breast cancer cells with high progesterone-receptor levels despite estrogen and antiestrogen resistance. Cell **28:**633–642.
36. **Hundertmark, S., H. Buhler, M. Rudolf, H. K. Weitzel, and V. Ragosch.** 1997. Inhibition of 11 beta-hydroxysteroid dehydrogenase activity enhances the antiproliferative effect of glucocorticosteroids on MCF-7 and ZR-75-1 breast cancer cells. J. Endocrinol. **155:**171–180.
37. **Joshi, P. A., H. W. Jackson, A. G. Beristain, M. A. Di Grappa, P. A. Mote, C. L. Clarke, J. Stingl, P. D. Waterhouse, and R. Khokha.** 2010. Progesterone induces adult mammary stem cell expansion. Nature **465:**803–807.
38. **Kato, S., et al.** 2005. Progesterone increases tissue factor gene expression, procoagulant activity, and invasion in the breast cancer cell line ZR-75-1. J. Clin. Endocrinol. Metab. **90:**1181–1188.
39. **Kelliher, M. A., D. C. Seldin, and P. Leder.** 1996. Tal-1 induces T cell acute lymphoblastic leukemia accelerated by casein kinase IIalpha. EMBO J. **15:** 5160–5166.
40. **Koyama, K., K. Myles, R. Smith, and Z. Krozowski.** 2001. Expression of the 11beta-hydroxysteroid dehydrogenase type II enzyme in breast tumors and modulation of activity and cell growth in PMC42 cells. J. Steroid Biochem. Mol. Biol. **76:**153–159.
41. **Landesman-Bollag, E., P. L. Channavajhala, R. D. Cardiff, and D. C. Seldin.** 1998. p53 deficiency and misexpression of protein kinase CK2alpha collaborate in the development of thymic lymphomas in mice. Oncogene **16:**2965–2974.
42. **Landesman-Bollag, E., et al.** 2001. Protein kinase CK2: signaling and tumorigenesis in the mammary gland. Mol. Cell. Biochem. **227:**153–165.
43. **Lange, C. A., T. Shen, and K. B. Horwitz.** 2000. Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome. Proc. Natl. Acad. Sci. U. S. A. **97:**1032–1037.
44. **Liston, P., et al.** 1996. Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. Nature **379:**349–353.
45. **Lupien, M., and M. Brown.** 2009. Cistromics of hormone-dependent cancer. Endocr. Relat. Cancer **16:**381–389.
46. **Lydon, J. P., et al.** 1995. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev. **9:**2266–2278.
47. **Lydon, J. P., G. Ge, F. S. Kittrell, D. Medina, and B. W. O'Malley.** 1999. Murine mammary gland carcinogenesis is critically dependent on progesterone receptor function. Cancer Res. **59:**4276–4284.
48. **Madak-Erdogan, Z., M. Lupien, F. Stossi, M. Brown, and B. S. Katzenellenbogen.** 2011. Genomic collaboration of estrogen receptor alpha and extracellular signal-regulated kinase 2 in regulating gene and proliferation programs. Mol. Cell. Biol. **31:**226–236.
49. **Meggio, F., and L. A. Pinna.** 2003. One-thousand-and-one substrates of protein kinase CK2? FASEB J. **17:**349–368.
50. **Migliaccio, A., et al.** 1998. Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. EMBO J. **17:** 2008–2018.
51. **Miro, F. A., et al.** 2002. Persistent nuclear accumulation of protein kinase CK2 during the G1-phase of the cell cycle does not depend on the ERK1/2 pathway but requires active protein synthesis. Arch. Biochem. Biophys. **406:** 165–172.
52. **Miyata, Y.** 2009. Protein kinase CK2 in health and disease: CK2: the kinase controlling the Hsp90 chaperone machinery. Cell. Mol. Life Sci. **66:**1840–1849.
53. **Mote, P. A., S. Bartow, N. Tran, and C. L. Clarke.** 2002. Loss of co-ordinate expression of progesterone receptors A and B is an early event in breast carcinogenesis. Breast Cancer Res. Treat. **72:**163–172.
54. **Mulac-Jericevic, B., J. P. Lydon, F. J. DeMayo, and O. M. Conneely.** 2003. Defective mammary gland morphogenesis in mice lacking the progesterone receptor B isoform. Proc. Natl. Acad. Sci. U. S. A. **100:**9744–9749.
55. **Murphy, L. C., L. J. Murphy, and R. P. Shiu.** 1988. Progestin regulation of EGF-receptor mRNA accumulation in T-47D human breast cancer cells. Biochem. Biophys. Res. Commun. **150:**192–196.
56. **Musgrove, E. A., C. S. Lee, and R. L. Sutherland.** 1991. Progestins both stimulate and inhibit breast cancer cell cycle progression while increasing expression of transforming growth factor alpha, epidermal growth factor receptor, c-fos, and c-myc genes. Mol. Cell. Biol. **11:**5032–5043.
57. **Narayanan, R., A. A. Adigun, D. P. Edwards, and N. L. Weigel.** 2005. Cyclin-dependent kinase activity is required for progesterone receptor function: novel role for cyclin A/Cdk2 as a progesterone receptor coactivator. Mol. Cell. Biol. **25:**264–277.
58. **Nardulli, A. M., and B. S. Katzenellenbogen.** 1988. Progesterone receptor regulation in T47D human breast cancer cells: analysis by density labeling of progesterone receptor synthesis and degradation and their modulation by progestin. Endocrinology **122:**1532–1540.
59. **Olsten, M. E., and D. W. Litchfield.** 2004. Order or chaos? An evaluation of the regulation of protein kinase CK2. Biochem. Cell Biol. **82:**681–693.
60. **Osborne, C. K., R. Schiff, G. Arpino, A. S. Lee, and V. G. Hilsenbeck.** 2005. Endocrine responsiveness: understanding how progesterone receptor can be used to select endocrine therapy. Breast **14:**458–465.
61. **Owen, G. I., J. K. Richer, L. Tung, G. Takimoto, and K. B. Horwitz.** 1998. Progesterone regulates transcription of the p21(WAF1) cyclin-dependent kinase inhibitor gene through Sp1 and CBP/p300. J. Biol. Chem. **273:**10696–10701.
62. **Pierson-Mullany, L. K., and C. A. Lange.** 2004. Phosphorylation of progesterone receptor serine 400 mediates ligand-independent transcriptional activity in response to activation of cyclin-dependent protein kinase 2. Mol. Cell. Biol. **24:**10542–10557.
63. **Resing, K. A., et al.** 1995. Determination of v-Mos-catalyzed phosphorylation sites and autophosphorylation sites on MAP kinase kinase by ESI/MS. Biochemistry **34:**2610–2620.
64. **Richer, J. K., et al.** 2002. Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells. J. Biol. Chem. **277:**5209–5218.
65. **Rothe, M., M. G. Pan, W. J. Henzel, T. M. Ayres, and D. V. Goeddel.** 1995. The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. Cell **83:**1243–1252.
66. **Sartorius, C. A., et al.** 1994. New T47D breast cancer cell lines for the independent study of progesterone B- and A-receptors; only antiprogestin-occupied B-receptors are switched to transcriptional agonists by cAMP. Cancer Res. **54:**3868–3877.
67. **Seagroves, T. N., J. P. Lydon, R. C. Hovey, B. K. Vonderhaar, and J. M. Rosen.** 2000. C/EBPbeta (CCAAT/enhancer binding protein) controls cell fate determination during mammary gland development. Mol. Endocrinol. **14:**359–368.
68. **Shen, T., K. B. Horwitz, and C. A. Lange.** 2001. Transcriptional hyperactivity of human progesterone receptors is coupled to their ligand-dependent down-regulation by mitogen-activated protein kinase-dependent phosphorylation of serine 294. Mol. Cell. Biol. **21:**6122–6131.
69. **Steeg, P. S., and Q. Zhou.** 1998. Cyclins and breast cancer. Breast Cancer Res. Treat. **52:**17–28.
70. **Stoecklin, E., M. Wissler, D. Schaetzle, E. Pfitzner, and B. Groner.** 1999. Interactions in the transcriptional regulation exerted by Stat5 and by members of the steroid hormone receptor family. J. Steroid Biochem. Mol. Biol. **69:**195–204.
71. **Takimoto, G. S., et al.** 1996. Role of phosphorylation on DNA binding and transcriptional functions of human progesterone receptors. J. Biol. Chem. **271:**13308–13316.
72. **Tawfic, S., et al.** 2001. Protein kinase CK2 signal in neoplasia. Histol. Histopathol. **16:**573–582.
73. **Trembley, J. H., Z. Chen, G. Unger, J. Slaton, B. T. Kren, C. Van Waes, and K. Ahmed.** 2010. Emergence of protein kinase CK2 as a key target in cancer therapy. Biofactors **36:**187–195.
74. **Trembley, J. H., G. Wang, G. Unger, J. Slaton, and K. Ahmed.** 2009. Protein kinase CK2 in health and disease: CK2: a key player in cancer biology. Cell. Mol. Life Sci. **66:**1858–1867.
75. **Ward, R. D., and N. L. Weigel.** 2009. Steroid receptor phosphorylation: assigning function to site-specific phosphorylation. Biofactors **35:**528–536.
76. **Weigel, N. L., et al.** 1995. Phosphorylation and progesterone receptor function. J. Steroid Biochem. Mol. Biol. **53:**509–514.
77. **Wilson, G. R., et al.** 2006. Activated c-SRC in ductal carcinoma in situ correlates with high tumour grade, high proliferation and HER2 positivity. Br. J. Cancer **95:**1410–1414.
78. **Yu, I. J., D. L. Spector, Y. S. Bae, and D. R. Marshak.** 1991. Immunocytochemical localization of casein kinase II during interphase and mitosis. J. Cell Biol. **114:**1217–1232.
79. **Zhang, Y., et al.** 1997. Phosphorylation of human progesterone receptor by cyclin-dependent kinase 2 on three sites that are authentic basal phosphorylation sites in vivo. Mol. Endocrinol. **11:**823–832.
80. **Zhang, Y., C. A. Beck, A. Poletti, D. P. Edwards, and N. L. Weigel.** 1994. Identification of phosphorylation sites unique to the B form of human progesterone receptor. In vitro phosphorylation by casein kinase II. J. Biol. Chem. **269:**31034–31040.
81. **Zhang, Z., C. Funk, D. Roy, S. Glasser, and J. Mulholland.** 1994. Heparin-binding epidermal growth factor-like growth factor is differentially regulated by progesterone and estradiol in rat uterine epithelial and stromal cells. Endocrinology **134:**1089–1094.

AUTHOR QUERY FORM

ELSEVIER	**Journal: MCE** **Article Number: 8037**	**Please e-mail or fax your responses and any corrections to:** **E-mail: corrections.esch@elsevier.sps.co.in** **Fax: +31 2048 52799**

Dear Author,

Please check your proof carefully and mark all corrections at the appropriate place in the proof (e.g., by using on-screen annotation in the PDF file) or compile them in a separate list. To ensure fast publication of your paper please return your corrections within 48 hours.

For correction or revision of any artwork, please consult http://www.elsevier.com/artworkinstructions.

Any queries or remarks that have arisen during the processing of your manuscript are listed below and highlighted by flags in the proof. Click on the 'Q' link to go to the location in the proof.

Location in article	Query / Remark: click on the Q link to go Please insert your reply or correction at the corresponding line in the proof
Q1	Please confirm that given names and surnames have been identified correctly.
Q2	Please check the insertion of keywords from metadatasheet.
Q3	This section comprises references that occur in the reference list but not in the body of the text. Please position each reference in the text or, alternatively, delete it. Any reference not dealt with will be retained in this section.
Q4	Please check Refs. 'Daniel et al. (xxxx), Daniel et al. (2010)' were identical instead of volume number and page range.
Q5	Please check the year in Ref. 'Daniel et al. (2010)'.
Q6	Please provide the year for the following refs. 'Daniel et al. (xxxx), Beguelin et al. (xxxx), Proietti et al. (xxxx), Cerliani et al. (xxxx), Santos et al. (xxxx), Jeong et al. (xxxx), Joshi et al. (xxxx).

Thank you for your assistance.

Molecular and Cellular Endocrinology 357 (2012) 43–49

Contents lists available at SciVerse ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Review

Role of phosphorylation in progesterone receptor signaling and specificity

Christy R. Hagan, Andrea R. Daniel, Gwen E. Dressing, Carol A. Lange *

University of Minnesota, Departments of Medicine and Pharmacology, Division of Hematology, Oncology, and Transplantation, Women's Cancer Program, Masonic Cancer Center, Minneapolis, MN 55455, United States

ARTICLE INFO

Article history:
Available online 16 September 2011

Keywords:
Progesterone receptor
Phosphorylation
Breast cancer
Promoter selectivity
Growth factors
Kinase

ABSTRACT

Progesterone receptors (PR), in concert with peptide growth factor-initiated signaling pathways, initiate massive expansion of the epithelial cell compartment associated with the process of alveologenesis in the developing mammary gland. PR-dependent signaling events also contribute to inappropriate proliferation observed in breast cancer. Notably, PR-B isoform-specific cross talk with growth factor-driven pathways is required for the proliferative actions of progesterone. Indeed, PRs act as heavily phosphorylated transcription factor "sensors" for mitogenic protein kinases that are often elevated and/or constitutively activated in invasive breast cancers. In addition, phospho-PR-target genes frequently include the components of mitogenic signaling pathways, revealing a mechanism for feed-forward signaling that confers increased responsiveness of, PR + mammary epithelial cells to these same mitogenic stimuli. Understanding the mechanisms and isoform selectivity of PR/kinase interactions may yield further insight into targeting altered signaling networks in breast and other hormonally responsive cancers (i.e. lung, uterine and ovarian) in the clinic. This review focuses on PR phosphorylation by mitogenic protein kinases and mechanisms of PR-target gene selection that lead to increased cell proliferation.

Contents

1. PR structure and function

The ovarian steroid hormone, progesterone, acts by binding to and activating progesterone receptor (PR) A-, B-, and C-isoforms expressed in target tissues. Isoform-specific expression results from selection of alternate promoters encoded by a single gene (Kastner et al., 1990). The full-length receptor, PR-B (116 kDa), contains a unique N-terminal segment, termed the B-upstream segment (BUS), that is not present in the truncated isoforms, PR-A (94 kDa), or PR-C (60 kDa). PR-C lacks both the BUS and a portion of the DNA-binding domain (DBD), rendering it transcriptionally inactive (Wei et al., 1996). In addition to intact DBDs, the two transcriptionally active isoforms, PR-B and PR-A, contain the following structural/functional domains: a flexible hinge region (H; also referred to as the carboxy terminal extension or CTE) that functions, in part, to aid DNA binding (Roemer et al., 2008), a ligand-binding domain (LBD), and multiple activating function (AF) domains required for transcriptional activity (Fig. 1). Studies from knockout-mice have shown that PR-B is necessary for the alveologenesis phase of normal mammary gland development, while PR-A is required for uterine development (Conneely et al., 2001; Lydon et al., 1995; Mulac-Jericevic et al., 2003; Shyamala et al., 1998). PR-C, lacking transcription activity, has been shown to inhibit PR-B function in the uterus (Condon et al., 2006), and conversely, appears to potentiate the transcriptional activity of the other PR isoforms in the breast (Wei et al., 1997).

* Corresponding author. Address: University of Minnesota, Departments of Medicine and Pharmacology, Division of Hematology, Oncology, and Transplantation, 420 Delaware St. SE, MMC 806, Minneapolis, MN 55455, United States. Tel.: +1 612 626 0621; fax: +1 612 626 4915.
E-mail address: Lange047@umn.edu (C.A. Lange).

0303-7207/$ - see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mce.2011.09.017

PR isoforms rapidly shuttle between the cytoplasm and the nucleus; unliganded receptors reside in both compartments and exist as part of multi-protein complexes in association with heat-shock protein chaperone molecules, such as Hsp70 and Hsp90 (Pratt et al., 1989; Kost et al., 1989). Additionally, unliganded and liganded PRs (primarily PR-B; (Boonyaratanakornkit et al., 2007)) participate in cytoplasmic or membrane-associated signaling complexes that activate mitogenic protein kinases, such as c-Src, MAPK and PI3 K (Boonyaratanakornkit et al., 2001; Migliaccio et al., 1998; Bagowski et al., 2001; Faivre and Lange, 2007; Carnevale et al.,

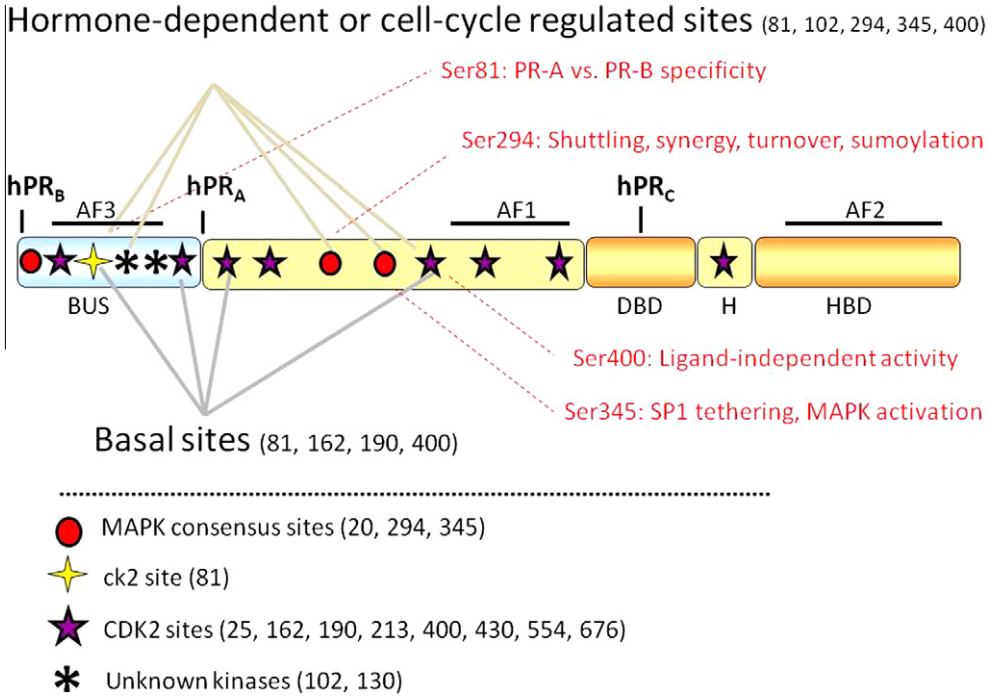

Fig. 1. Progesterone receptor structure and phosphorylation sites. All three human PR isoforms (hPRA, hPRB and hPRC) are transcribed from the same gene, containing distal and proximal promoters. Shown are three transcription activation function (AF) domains, the B-upstream segment (BUS), the DNA-binding domain (DBD), the hinge region (H) and the hormone-binding domain (HBD). PR is phosphorylated basally, as well as in response to hormone. Shown here are known PR phosphorylation sites as determined in vitro and in vivo, and the protein kinases that are likely responsible for direct phosphorylation at these sites.

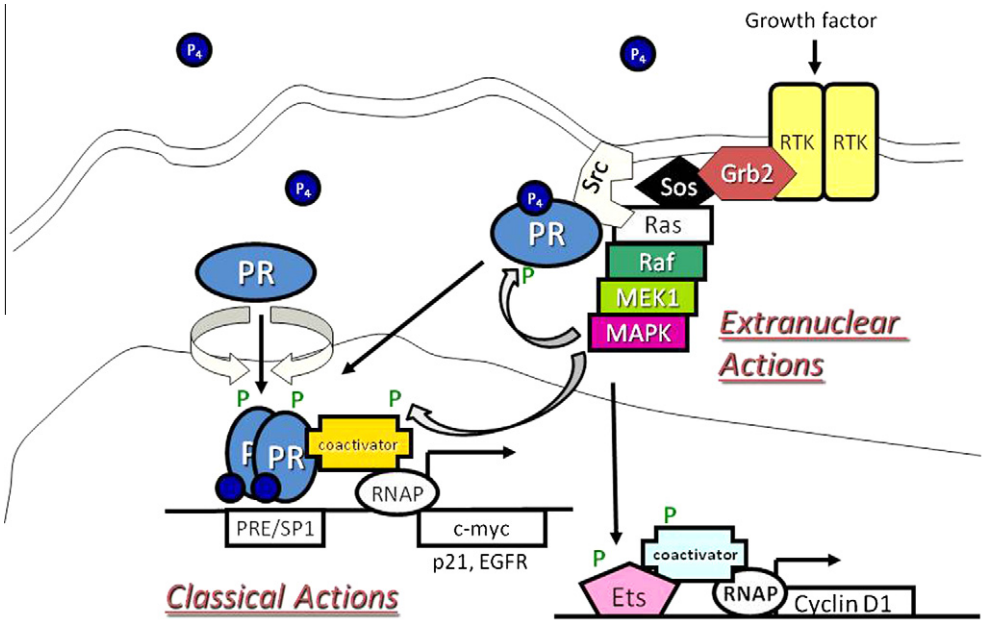

Fig. 2. Integration of PR rapid signaling and transcriptional activities. Progesterone (P4) binding to PR induces the rapid association of PR and c-Src. This interaction leads to a c-Src-dependent activation of the MAPK module through Ras/Raf signaling. This MAPK activation can lead to phosphorylation (P) of PR, transcriptional coactivators, and/or activation of downstream MAPK target genes (i.e. Cyclin D1). Phosphorylated PRs can activate transcription directly by binding to progesterone response elements (PREs) or indirectly though tethering interactions (i.e. SP1). Extranuclear and classical actions of PR are likely highly integrated actions, rather than separable events mediated by discrete populations of receptors.

2007). In response to progesterone-binding, membrane-tethered PRs rapidly activate these kinases and can also transactivate EGFR (Faivre et al., 2008); this PR-dependent activity has been termed a "non-genomic" action because it occurs independently of the transcriptional activity of PRs (Fig. 2). In the classical or genomic model of PR action, ligand binding induces dissociation of PR from chaperone complexes; dimerized (hetero or homo) PRs are largely retained in the nucleus where they bind to DNA either directly through progesterone response elements (PRE), or indirectly through tethering interactions with other transcription factors (AP1, SP1, STATs) (Owen et al., 1998; Stoecklin et al., 1999; Cicatiello et al., 2004).

Notably, PR-A and PR-B isoforms are highly post-translationally modified, primarily on serine (Ser; phosphorylation) and lysine (Lys; acetylation, ubiquitination, and sumoylation) residues located in the N-terminal region (Lange et al., 2000; Weigel et al., 1995; Daniel et al., 2010; Abdel-Hafiz et al., 2002; Hagan et al., 2011). These modifications are frequently ligand-dependent, but can also occur independently of progestin-binding (i.e. in response to kinase activation), and significantly alter receptor stability, localization, tethering interactions, transcriptional activity, and promoter selectivity (Daniel et al., 2009; Ward and Weigel, 2009). For example, PR phospho-species exhibit differential activities on a given promoter, but also appear to select different promoters (reviewed in (Daniel et al., 2009)). Although the mechanisms are not entirely clear, unique PR phospho-species are likely directed to distinct PR target gene subsets in part via phosphorylation-dependent protein–protein interactions with the same set of protein kinases that may occur within so-called "rapid signaling complexes". Thus, the non-genomic and genomic actions of PRs are highly integrated functions that serve to coordinate isoform-specific PR actions and mediate PR-target gene promoter selection; mechanisms of PR integration with signaling pathways is the topic of this review (Fig. 2).

2. MAPK activation alters PR function

MAPK signaling modulates PR activity directly by phosphorylating the receptor on consensus site serine residues Ser294 and Ser345 (Faivre et al., 2008; Lange et al., 2000). These distinctly regulated phosphorylation events have unique functional consequences for PR that ultimately regulate cell fate. Upon growth factor stimulation, PR phosphorylation of Ser294 primes the receptor for robust transcriptional activation in response to ligand by ensuring retention in the nucleus (discussed below) (Qiu et al., 2003), association with DNA (Daniel et al., 2007a,b), and removal of repressive modification by sumoylation (Daniel et al., 2007b). Ser294-phosphorylated receptors are transcriptionally hypersensitive to low concentrations of ligand on a select set of promoters (Qiu and Lange, 2003); the mechanism of growth factor-induced PR hypersensitivity maps to phospho-Ser294 antagonism of Lys388 sumoylation (Daniel et al., 2007a,b). Likewise, phosphorylation on Ser294 increases PR ubiquitinylation, an activation step for many transcription factors (Salghetti et al., 2001), and also augments its downregulation (Lange et al., 2000). Therefore Ser294 phosphorylation in response to MAPK activation (by either progestins or growth factors) generates receptors that are hyperactive on select promoters in response to ligand and serves to couple this activity to rapid proteasome-dependent turnover. In addition, phosphorylated/desumoylated receptors are active on a subset of ligand-independent PR-target gene promoters whose expressed protein products (IRS-1 and STC1) contribute to breast cancer cell proliferation and pro-survival (Daniel and Lange, 2009). Furthermore, Ser294 appears to be a "hot-spot" for the regulation of PR-B transcriptional activity as phospho-mutant PR-B (Ser294 to alanine) is virtually transcriptionally inactive when measured on endogenous genes (i.e. in cells stably expressing S294A PR-B relative to cells containing wild-type PR) (Shen et al., 2001). Notably, PR-A is not appreciably phosphorylated on Ser294 in intact cells, while this site in PR-A can be phosphorylated *in vitro* using recombinant PR-A proteins (Clemm et al., 2000). This finding underscores the role of protein–protein interactions between PRs and associated signaling complexes that contain protein kinases as major determinants of PR isoform specificity.

In an alternative route to phosphorylation-dependent PR promoter selection, rapid progestin-mediated MAPK activation drives specific phosphorylation of PR-B on Ser345, a site shown to be critical for PR tethering to SP1 transcription factors (Faivre et al., 2008). PR/c-Src/EGFR rapid signaling complex formation precedes PR Ser345 phosphorylation and PR/SP1 association with non-classical promoters (lacking PREs), such as p21 and EGFR (Fig. 2). This unique mechanism of steroid receptor activation by MAPK signaling (i.e. non-genomic/genomic signaling integration) is required for progestin-induced breast cancer cell entry into S-phase (Faivre et al., 2008).

3. Cyclin dependent kinase 2 (CDK2) regulation of PR function

Studies using both *in vitro* and *in vivo* techniques have identified multiple CDK2-dependent phosphorylation sites on PR (reviewed in (Moore et al., 2007)). These sites include PR serines 25, 162, 190, 213, 400, 554, 676 (Zhang et al., 1997; Knotts et al., 2001) and threonine 430 (Knotts et al., 2001). Additionally, while Ser294 is phosphorylated by MAPK (discussed earlier), it can also be phosphorylated by CDK2 (Daniel and Lange, 2009). Although only a fraction of these CDK2 sites have been studied in depth, PR phosphorylation by CDK2 has specific implications for PR function and activity. Phosphorylation of PR on serines 190, 294, 554 and 676 clearly contributes to PR hormone-dependent transcriptional activity (Shen et al., 2001; Takimoto et al., 1996). Individual mutation of each of these sites results in significant decreases (20–90%) in overall PR transcriptional activity, as measured using PRE-reporter gene constructs. While Sers 190, 554 or 676- phospho-mutant PRs exhibit significant decreases in transcriptional activity, these mutant PR species are each able to bind DNA similarly to wild-type PR, suggesting that phosphorylation at these serines may contribute to recruitment of co-activators to PR-containing transcriptional complexes (Takimoto et al., 1996).

Phosphorylation of PR Ser400 by CDK2 has been linked to enhanced ligand-independent PR transcriptional activity, as measured using PRE-reporter gene constructs (Pierson-Mullany and Lange, 2004). In the presence of high CDK2 kinase activity and/or low cell cycle inhibitors (namely, p27), PR Ser400 is constitutively phosphorylated and thereby drives heightened PR transcriptional activity in the absence of progestins (Pierson-Mullany and Lange, 2004). This particular interaction of CDK2 with PR has important implications for deregulated PR activity in the context of breast cancer, as transformed cells often exhibit loss of cell cycle control that is characterized by Rb-inactivation, elevated CDK4/6 activity, high expression of cyclins D, E, or A, and/or low expression of cell cycle inhibitors (Slingerland and Pagano, 2000; Cariou et al., 1998; Musgrove et al., 2004; Alkarain et al., 2004; Tawfic et al., 2001; Wilson et al., 2006) ultimately leading to increased, deregulated CDK2 activity. Notably, PR-target genes include key cell cycle mediators (reviewed in (Dressing and Lange, 2009)) such as D-type cyclins and cyclin E, the regulatory subunits of CDK4/6 and CDK2, respectively. Thus, activation of unliganded PRs in this setting (cell cycle deregulation leading to high CDK2 activity) may produce a "feed forward" mechanism of persistent CDK2 activation early in breast tumor development. This unliganded activity of PR can be

blocked by anti-progestins (Pierson-Mullany and Lange, 2004), suggesting that selective PR modulators could be used to block CDK2-driven cell proliferation and pro-survival in PR + tumor cells.

Phosphorylation events also contribute to PR nuclear localization. Recent studies suggest that mutant PRs unable to enter the nucleus (devoid of nuclear localizations signals; ΔNLS PR) are phosphorylated on Ser190, but not on Sers 81, 294, 345 and 400 ((Daniel et al., 2010) and data not shown). However, ΔNLS PR is phosphorylated on these sites upon coexpression and dimerization with wt PR, forcing ΔNLS PR nuclear entry. Other studies showed that Ser400 phosphorylation (CDK2-dependent) enhanced ligand-induced nuclear accumulation (Pierson-Mullany and Lange, 2004), while Ser294 phosphorylation was required for growth factor (EGF, MAPK), but not progestin-mediated nuclear accumulation (Qiu et al., 2003). These data suggest that phosphorylation of PR on these residues occurs upon nuclear entry and serves to promote nuclear retention. Phosphorylation on Ser190, another CDK2 site, likely occurs in the cytoplasm and does not contribute to nuclear entry or retention (Daniel et al., 2010). Together, these data demonstrate that CDK2 is able to phosphorylate PR in both the cytoplasm and the nucleus and that, once in the nucleus, phosphorylation at some CDK2 sites (Sers 294 and 400) promotes PR nuclear retention, perhaps via protein–protein interactions requiring these specific phosphorylation events. Moreover, rapid nuclear translocation and retention of PR appears to be critical for proper execution of rapidly-activated (i.e. c-myc mRNA expression is induced by liganded PR within minutes) PR-target genes (Daniel et al., 2010). Properly timed PR nuclear entry/retention in response to phosphorylation events likely ensures robust execution of PR transcriptional activity at such "early genes" perhaps, in part, by ensuring that both PR and its co-regulators are activated (i.e. appropriately phosphorylated) and co-localized in the nucleus. Indeed, latent nuclear localization of PR is associated with delays in PR-induced immediate early genes (i.e. c-myc) but not in overall PR transcriptional activity measured at late time points (i.e. on reporter genes) and/or on endogenous genes that are not particularly sensitive to changes in phosphorylation events (Daniel et al., 2010).

Interestingly, CDK2 not only acts to phosphorylate PR but may also act as an integral part of PR transcriptional complexes. Cyclins A and E, the regulatory subunits of CDK2, bind to both unliganded and liganded PRs; these constitutive interactions may serve to recruit and sustain CDK2 activity at active sites of transcription (reviewed in (Dressing and Lange, 2009)). Although endogenous genes have not been extensively studied, Cyclin A is clearly recruited along with PR to stably embedded (i.e. in chromatin) MMTV promoter regions (Narayanan et al., 2005). Thus, CDK2 (a cyclin A-binding partner) is also likely present at PR-bound PRE-containing enhancers (Moore et al., 2007; Narayanan et al., 2005; Weigel and Moore, 2007). Inhibition of CDK2 activity using a small molecule CDK2 inhibitor, roscovitine, decreased phosphorylation of SRC-1 (steroid receptor co-activator-1) and blocked recruitment of both PR and SRC-1 to the PR transcriptional complex on the MMTV promoter (Narayanan et al., 2005). In these studies, mutation of PR at multiple CDK2 phosphorylation sites had no effect on reporter gene transcription. Thus, CDK2 appears to mediate SRC-1 co-activator phosphorylation (independently of PR phosphorylation). The scaffolding function of PR/cyclin interactions likely serves to recruit and sustain CDK2 activity (wherein the primary substrate is SRC-1); this model awaits confirmation on endogenous genes and during cell cycle traverse.

4. ck2 modification of PR

Initial *in vitro* work showed that PR Ser81 (unique to the BUS region of PR-B) was phosphorylated by ck2, a ubiquitously expressed, constitutively active protein kinase (Zhang et al., 1994). Recent published work from the Lange lab has shown in breast cancer cells that basal levels of PR Ser81 phosphorylation are rapidly increased in response to either agonist or antagonist ligands (Hagan et al., 2011); an effect shown to be dependent on ck2. However, unlike other PR phosphorylation sites (i.e. Ser294), PR Ser81 phosphorylation is unresponsive to growth-factor or serum treatment of cells. Interestingly, in the absence of ligand, PR Ser81 phosphorylation is increased in cells that are synchronized at the G1/S phase border, suggesting that phosphorylation at this site is regulated in a cell-cycle dependent manner (Hagan et al., 2011). In line with this finding, ligand-independent cell survival, as measured by soft-agar colony formation, was decreased in cells expressing a PR phosphomutant (S79/81A PR) that cannot be phosphorylated at Ser81 (Hagan et al., 2011). Moreover, this mutant displayed defects in recruitment to selected PR-B-target genes important for proliferation and pro-survival, and was impaired in its ability to recruit ck2 to PR-associated enhancer sites (Fig. 3) (Hagan et al., 2011). ck2, a kinase shown to be upregulated in every cancer studied thus far, including breast cancer, is not thought to be oncogenic on its own, but appears to increase the oncogenic potential of cancer-promoting proteins and pro-growth signals that are its substrate molecules (Tawfic et al., 2001; Trembley et al., 2009). In the context of breast cancer, where progestins have been implicated as a risk factor for tumor development and early progression (Beral, 2003; Anderson et al., 2004; Chlebowski et al., 2003), overexpressed ck2 could further enhance the oncogenic potential of PR through inappropriate phosphorylation (on Ser81), thereby directing phospho-Ser81 PR-B to growth-promoting genes.

5. PR-dependent activation and amplification of kinase signaling pathways

Several studies illustrate the emerging concept that PR and associated signaling pathways are fully integrated, from membrane-initiated events to genomic actions (Fig. 2). Upon progestin treatment, PR rapidly associates with signaling complexes via two distinct domains: a consensus poly-proline rich region (PR amino acids 396–456) known to interact with consensus SH3 domains (Boonyaratanakornkit et al., 2001) and unique (to PR) regions termed Estrogen Receptor Interacting Domains or ERIDs; ERID1 (amino acids 165–345) and ERID2 (amino acids 456–546) are located in the PR N-terminus (Ballare et al., 2003). Progestin-binding induces direct interaction of PR with the SH3 domain of c-Src, or to ER (via the ERID domains), causing rapid (5–10 min) activation of the EGFR/c-Src/Ras/Erk pathway (Boonyaratanakornkit et al., 2001; Migliaccio et al., 1998; Faivre et al., 2008; Ballare et al., 2003) and the PI3K/Akt pathway (Carnevale et al., 2007). These signals, shown to be critical for progestin-induced proliferation of breast cancer cells (Boonyaratanakornkit et al., 2007), provide a feed forward signaling mechanism for PR/progestin-dependent genomic events in addition to activating other transcription factors (Faivre et al., 2008). Phosphorylation of PR and co-activator molecules enhances PR transcriptional activity on classical (Qiu and Lange, 2003) and non-classical promoters (Faivre et al., 2008). Progestin-activated Erk is recruited to PR-containing transcriptional complexes in chromatin (Vicent et al., 2006) and PR devoid of ERIDs activates a gene expression profile distinct from wt PR (Quiles et al., 2009), indicating that PR-induced kinase signaling contributes directly to promoter activation and selectivity. Notably, progestin treatment also elicits delayed (18 h) and sustained activation of MAPK signaling, whereby MAPK-dependent upregulation of PR-target genes (Wnt1, MMPs, and EGFR) completes an autocrine signaling pathway that culminates in high cyclin D levels and breast cancer cell growth/survival in soft agar (Faivre and Lange,

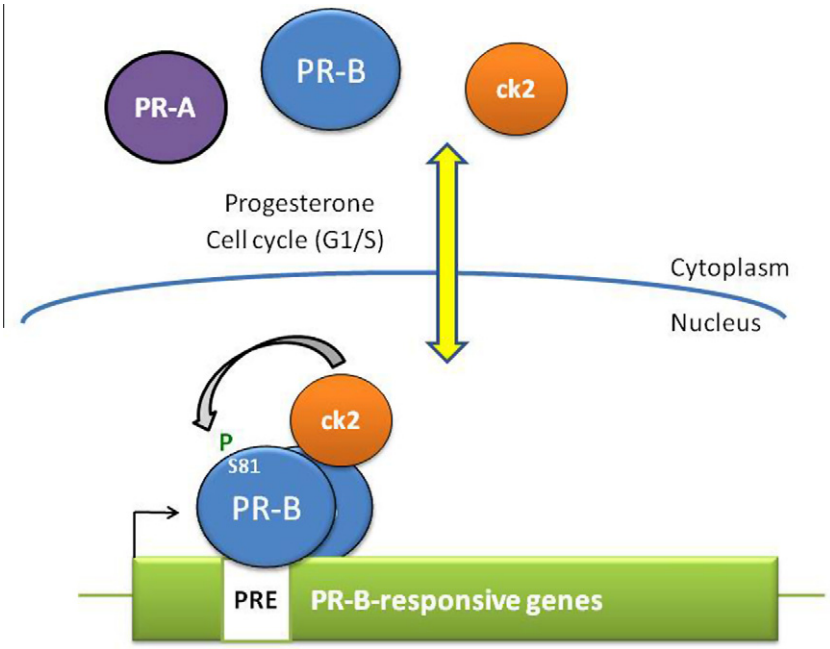

Fig. 3. Ck2-dependent PR-B Ser81 phosphorylation mediates isoform-specfic target gene selection. In response to progesterone binding or cell cycling (G1/S), PR-B is phosphorylated at Ser81 by ck2. Phospho-Ser81-PR-B/ck2 complexes are recruited to promoter/enhancer regions of Ser81-responsive PR-target genes. Phosphorylation at PR-B Ser81 (not present in PR-A) is a major determinant of PR isoform-specific target-gene selectivity.

2007). Thus, progestin/PR-mediated rapid activation of MAPK signaling ultimately functions to amplify PR genomic actions, modulate PR target gene selectivity (i.e. by directing phospho-PRs to selected promoters), and induce sustained MAPK signaling (i.e. downstream of activated EGFR) capable of activating multiple (PR-independent) transcription factors that serve to perpetuate the proliferative signal (long after liganded PRs have been downregulated). In this manner, progesterone/PRs may confer greatly increased sensitivity of target tissues to the actions of peptide growth factors. These interactions clearly allow for rapid expansion of the mammary epithelium during puberty and pregnancy (in preparation for lactation), but may inappropriately drive early breast cancer progression of steroid hormone receptor positive tumors.

In addition to scaffolding MAPK pathway signaling events, PR also participates in signaling complexes with cell cycle regulators. PR contains numerous consensus CDK binding motifs, and has been shown to associate with CDK2, perhaps mediating its interactions with cyclins E and A (discussed above) (Narayanan et al., 2005; Faivre et al., 2005). This complex formation, in addition to PR transcriptional upregulation of cyclins and CDK inhibitors (p21, p27) that appears to be required for initiating CDK kinase activity, may account for the rapid (15 min) and sustained (days) activation of CDK2 observed in breast cancer cells upon a single treatment with progestin (Pierson-Mullany and Lange, 2004). Again, these studies indicate that phospho-PRs are capable of robust positive feed forward or self-regulation of the very same signaling pathways that they rapidly activate.

A number of studies have illustrated further cross-talk between PRs and Signal Transducers and Activators of Transcription (STATs), involving PR-mediated activation of both STAT3 and STAT5. Cumulative work from the Elizalde lab has shown that STAT3 activation by the heregulin/ErbB-2 pathway is mediated by ligand-independent functions of PR, and requires phosphorylation of PR Ser294 (in response to growth factor stimulation) (Proietti et al., 2009). Further work has defined a bi-directional transcriptional co-activator relationship between PR and STAT3, each appearing to activate the transcriptional capacity of the other (Beguelin et al., 2010; Proietti et al., 2010). A similar story has emerged for STAT5 and PR. Progesterone treatment induces PR-dependent STAT5 nuclear translocation and transcriptional activity, potentially mediated by a direct interaction between PR and STAT5 (Richer et al., 1998), at times involving other signaling molecules that serve as co-regulators like FGFR-2 (Fibroblast growth factor receptor-2) (Cerliani et al., 2011). PR-dependent regulation of (downstream) STAT5 activity is well established as critical for normal mammary gland development (Santos et al., 2010; Santos et al., 2008).

Indeed, the end point of mitogenic signaling pathway activation is often the regulation of transcription factor substrates. Notably, phospho-PR target genes most often include the components of signal transduction pathways (T. Knutson and C. Lange, unpublished results). Thus, PR is directly responsible for modulating/maintaining kinase signaling in cells via transcriptional upregulation of growth factor receptors, their ligands, and their downstream effectors and associated adaptor molecules. Direct PR target genes include EGFR, IRS1, STAT5A, numerous Ras pathway members (including adaptors and exchange factors), many kinases, as well as peptide growth factors (Hb-EGF, Wnt1) and other secreted signaling molecules (Daniel et al., 2007a,b; Jacobsen et al., 2003). Ultimately, kinase pathway "restructuring" by PR may serve to prime mammary epithelial cells for the rapid proliferation stage associated with massive expansion of the (pregnant) mammary gland that occurs in preparation for lactation. Similarly, the deregulation of these events during breast cancer development and/or early progression is suspected to contribute to advanced malignant breast cancer phenotypes.

6. PR significance in breast cancer

Highly publicized and controversial clinical data has demonstrated that women taking hormone-replacement therapy (HRT) whose regimens included estrogen and synthetic progesterone, but not estrogen alone, experienced increased breast tumor number, size, and aggressiveness (Beral, 2003; Anderson et al., 2004) increased breast cancer risk was reversed upon cessation of HRT

(Beral, 2003; Chlebowski et al., 2009). Significantly, nearly 70% of breast cancers express both ER and PR at diagnosis, in contrast to PR/ER expression in just 7–10% of normal (non-pregnant) breast luminal epithelium (Seagroves et al., 2000). As these steroid receptor (SR)-positive tumors progress, many of them become hormone-independent (refractory to estrogen- or ER-targeted endocrine treatments) while retaining high SR expression, suggesting an early switch to autocrine and/or paracrine growth factor signaling (Osborne et al., 2005). In addition, a majority of these cancers have upregulated and activated protein kinases, such as MAPK, Akt, c-Src, cyclin/CDKs, and ck2, all of which modify and/or activate PR and/or its co-regulators (discussed in detail above) (Tawfic et al., 2001; Wilson et al., 2006; Gregory et al., 2004; Steeg and Zhou, 1998). In breast cancer cells, PR-B action clearly drives proliferation and pro-survival signaling. Interestingly, PR (mRNA expression) was recently identified as an independent-(single-gene) predictor of poor outcome in non-small cell lung cancer, implicating PR and hormone-responsiveness in cancers other than breast (Jeong et al., 2010). In an environment where progesterone is no longer required to drive cellular proliferation (i.e. ligand-independence), constitutive activation of PR-activating protein kinases may promote uncontrolled cell growth that is primarily driven by deregulated phospho-PR-target genes. Most recently, progesterone was shown to mediate mammary gland stem cell self-renewal via paracrine mechanisms in which secreted factors (Wnt, RANKL) derived from PR-positive cells influenced the PR-null stem cell niche (Joshi et al., 2010; Asselin-Labat et al., 2006). Progesterone/progestins may alter breast cancer stem cell behavior by similar mechanisms. In sum, in light of the cumulative data discussed herein, understanding how mitogenic protein kinases alter PR (and vice versa) is critical to fully understanding breast tumor etiology with the goal of developing superior approaches for the prevention or treatment of endocrine resistance in SR-positive breast cancers.

References

Kastner, P., Krust, A., Turcotte, B., Stropp, U., Tora, L., Gronemeyer, H. and Chambon, P., 1990. Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B, Embo J. 9.

Wei, L.L., Hawkins, P., Baker, C., Norris, B., Sheridan, P.L., Quinn, P.G., 1996. An amino-terminal truncated progesterone receptor isoform, PRc, enhances progestin-induced transcriptional activity. Mol. Endocrinol. 10, 1379–1387.

Roemer, S.C., Adelman, J., Churchill, M.E., Edwards, D.P., 2008. Mechanism of high-mobility group protein B enhancement of progesterone receptor sequence-specific DNA binding. Nucleic Acids Res. 36, 3655–3666.

Conneely, O.M., Mulac-Jericevic, B., Lydon, J.P., De Mayo, F.J., 2001. Reproductive functions of the progesterone receptor isoforms: lessons from knock-out mice. Mol. Cell Endocrinol. 179, 97–103.

Lydon, J.P., DeMayo, F.J., Funk, C.R., Mani, S.K., Hughes, A.R., Montgomery, C.A., Shyamala, G., Conneely, O.M., O'Malley, B.W., 1995. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev. 9, 2266–2278.

Mulac-Jericevic, B., Lydon, J.P., DeMayo, F.J., Conneely, O.M., 2003. Defective mammary gland morphogenesis in mice lacking the progesterone receptor B isoform. Proc. Natl. Acad Sci. USA 100, 9744–9749.

Shyamala, G., Yang, X., Silberstein, G., Barcellos-Hoff, M.H., Dale, E., 1998. Transgenic mice carrying an imbalance in the native ratio of A to B forms of progesterone receptor exhibit developmental abnormalities in mammary glands. Proc. Natl. Acad. Sci. USA 95, 696–701.

Condon, J.C., Hardy, D.B., Kovaric, K., Mendelson, C.R., 2006. Up-regulation of the progesterone receptor (PR)-C isoform in laboring myometrium by activation of nuclear factor-kappaB may contribute to the onset of labor through inhibition of PR function. Mol. Endocrinol. 20, 764–775.

Wei, L.L., Norris, B.M., Baker, C.J., 1997. An N-terminally truncated third progesterone receptor protein, PR(C), forms heterodimers with PR(B) but interferes in PR(B)-DNA binding. J. Steroid Biochem. Mol. Biol. 62, 287–297.

Pratt, W.B., Sanchez, E.R., Bresnick, E.H., Meshinchi, S., Scherrer, L.C., Dalman, F.C., Welsh, M.J., 1989. Interaction of the glucocorticoid receptor with the Mr 90, 000 heat shock protein: an evolving model of ligand-mediated receptor transformation and translocation. Cancer Res. 49, 2222s–2229s.

Kost, S.L., Smith, D.F., Sullivan, W.P., Welch, W.J., Toft, D.O., 1989. Binding of heat shock proteins to the avian progesterone receptor. Mol. Cell Biol. 9, 3829–3838.

Boonyaratanakornkit, V., McGowan, E., Sherman, L., Mancini, M.A., Cheskis, B.J., Edwards, D.P., 2007. The role of extranuclear signaling actions of progesterone receptor in mediating progesterone regulation of gene expression and the cell cycle. Mol. Endocrinol. 21, 359–375.

Boonyaratanakornkit, V., Scott, M.P., Ribon, V., Sherman, L., Anderson, S.M., Maller, J.L., Miller, W.T., Edwards, D.P., 2001. Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases. Mol. Cell. 8, 269–280.

Migliaccio, A., Piccolo, D., Castoria, G., Di Domenico, M., Bilancio, A., Lombardi, M., Gong, W., Beato, M., Auricchio, F., 1998. Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. Embo J. 17, 2008–2018.

Bagowski, C.P., Myers, J.W., Ferrell, J.E., 2001. The classical progesterone receptor associates with p42 MAPK and is involved in PI3-K signaling in *Xenopus* oocytes. J. Biol. Chem. 276, 37708–37714.

Faivre, E.J., Lange, C.A., 2007. Progesterone receptors upregulate Wnt-1 to induce epidermal growth factor receptor transactivation and c-Src-dependent sustained activation of Erk1/2 mitogen-activated protein kinase in breast cancer cells. Mol. Cell Biol. 27, 466–480.

Carnevale, R.P., Proietti, C.J., Salatino, M., Urtreger, A., Peluffo, G., Edwards, D.P., Boonyaratanakornkit, V., Charreau, E.H., Bal de Kier Joffe, E., Elizalde, P.V., Schillaci, R., 2007. Progestin effects on breast cancer cell proliferation, proteases activation, and in vivo development of metastatic phenotype all depend on progesterone receptor capacity to activate cytoplasmic signaling pathways. Mol. Endocrinol. 21, 1335–1358.

Faivre, E.J., Daniel, A.R., Hillard, C.J., Lange, C.A., 2008. Progesterone receptor rapid signaling mediates serine 345 phosphorylation and tethering to specificity protein 1 transcription factors. Mol. Endocrinol. 22, 823–837.

Owen, G.I., Richer, J.K., Tung, L., Takimoto, G., Horwitz, K.B., 1998. Progesterone regulates transcription of the p21(WAF1) cyclin-dependent kinase inhibitor gene through Sp1 and CBP/p300. J. Biol. Chem. 273, 10696–10701.

Stoecklin, E., Wissler, M., Schaetzle, D., Pfitzner, E., Groner, B., 1999. Interactions in the transcriptional regulation exerted by Stat5 and by members of the steroid hormone receptor family. J. Steroid Biochem. Mol. Biol. 69, 195–204.

Cicatiello, L., Addeo, R., Sasso, A., Altucci, L., Petrizzi, V.B., Borgo, R., Cancemi, M., Caporali, S., Caristi, S., Scafoglio, C., Teti, D., Bresciani, F., Perillo, B., Weisz, A., 2004. Estrogens and progesterone promote persistent CCND1 gene activation during G1 by inducing transcriptional derepression via c-Jun/c-Fos/estrogen receptor (progesterone receptor) complex assembly to a distal regulatory element and recruitment of cyclin D1 to its own gene promoter. Mol. Cell Biol. 24, 7260–7274.

Lange, C.A., Shen, T., Horwitz, K.B., 2000. Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome. Proc. Natl. Acad. Sci. USA 97, 1032–1037.

Weigel, N.L., Bai, W., Zhang, Y., Beck, C.A., Edwards, D.P., Poletti, A., 1995. Phosphorylation and progesterone receptor function. J. Steroid Biochem. Mol. Biol. 53, 509–514.

Daniel, A.R., Gaviglio, A.L., Czaplicki, L.M., Hillard, C.J., Housa, D., Lange, C.A., 2010. The Progesterone Receptor Hinge Region Regulates the Kinetics of Transcriptional Responses Through Acetylation, Phosphorylation, and Nuclear Retention. Mol. Endocrinol. 24, 2126–2138.

Abdel-Hafiz, H., Takimoto, G.S., Tung, L., Horwitz, K.B., 2002. The inhibitory function in human progesterone receptor N termini binds SUMO-1 protein to regulate autoinhibition and transrepression. J. Biol. Chem. 277, 33950–33956.

Hagan, C.R., Regan, T.M., Dressing, G.E., Lange, C.A., 2011. Ck2-dependent phosphorylation of progesterone receptors (pr) on ser81 regulates pr-b isoform-specific target gene expression in breast cancer cells. Mol. Cell Biol. 31, 2439–2452.

Daniel, A.R., Knutson, T.P., Lange, C.A., 2009. Signaling inputs to progesterone receptor gene regulation and promoter selectivity. Mol. Cell Endocrinol. 308, 47–52.

Ward, R.D., Weigel, N.L., 2009. Steroid receptor phosphorylation: Assigning function to site-specific phosphorylation. Biofactors 35, 528–536.

Qiu, M., Olsen, A., Faivre, E., Horwitz, K.B., Lange, C.A., 2003. Mitogen-activated protein kinase regulates nuclear association of human progesterone receptors. Mol. Endocrinol. 17, 628–642.

Daniel, A.R., Qiu, M., Faivre, E.J., Ostrander, J.H., Skildum, A., Lange, C.A., 2007a. Linkage of progestin and epidermal growth factor signaling: phosphorylation of progesterone receptors mediates transcriptional hypersensitivity and increased ligand-independent breast cancer cell growth. Steroids 72, 188–201.

Daniel, A.R., Faivre, E.J., Lange, C.A., 2007b. Phosphorylation-dependent antagonism of sumoylation derepresses progesterone receptor action in breast cancer cells. Mol. Endocrinol. 21, 2890–2906.

Qiu, M., Lange, C.A., 2003. MAP kinases couple multiple functions of human progesterone receptors: degradation, transcriptional synergy, and nuclear association. J. Steroid Biochem. Mol. Biol. 85, 147–157.

Salghetti, S.E., Caudy, A.A., Chenoweth, J.G., Tansey, W.P., 2001. Regulation of transcriptional activation domain function by ubiquitin. Science 293, 1651–1653.

Daniel, A.R., Lange, C.A., 2009. Protein kinases mediate ligand-independent derepression of sumoylated progesterone receptors in breast cancer cells. Proc. Natl. Acad Sci. USA 106, 14287–14292.

Shen, T., Horwitz, K.B., Lange, C.A., 2001. Transcriptional hyperactivity of human progesterone receptors is coupled to their ligand-dependent down-regulation by mitogen-activated protein kinase-dependent phosphorylation of serine 294. Mol. Cell Biol. 21, 6122–6131.

Clemm, D.L., Sherman, L., Boonyaratanakornkit, V., Schrader, W.T., Weigel, N.L., Edwards, D.P., 2000. Differential hormone-dependent phosphorylation of progesterone receptor A and B forms revealed by a phosphoserine site-specific monoclonal antibody. Mol Endocrinol. 14, 52–65.

Moore, N.L., Narayanan, R., Weigel, N.L., 2007. Cyclin dependent kinase 2 and the regulation of human progesterone receptor activity. Steroids 72, 202–209.

Zhang, Y., Beck, C.A., Poletti, A., Clement, J.P.t., Prendergast, P., Yip, T.T., Hutchens, T.W., Edwards, D.P., Weigel, N.L., 1997. Phosphorylation of human progesterone receptor by cyclin-dependent kinase 2 on three sites that are authentic basal phosphorylation sites in vivo. Mol. Endocrinol. 11, 823–832.

Knotts, T.A., Orkiszewski, R.S., Cook, R.G., Edwards, D.P., Weigel, N.L., 2001. Identification of a phosphorylation site in the hinge region of the human progesterone receptor and additional amino-terminal phosphorylation sites. J. Biol. Chem. 276, 8475–8483.

Takimoto, G.S., Hovland, A.R., Tasset, D.M., Melville, M.Y., Tung, L., Horwitz, K.B., 1996. Role of phosphorylation on DNA binding and transcriptional functions of human progesterone receptors. J. Biol. Chem. 271, 13308–13316.

Pierson-Mullany, L.K. and Lange, C.A., 2004. Phosphorylation of Progesterone Receptor Serine 400 Mediates Ligand-Independent Transcriptional Activity in Response to Activatoin of Cyclin-Dependent Protein Kinase2, Mol. Cell. Biol. 24.

Slingerland, J., Pagano, M., 2000. Regulation of the cdk inhibitor p27 and its deregulation in cancer. J. Cell Physiol. 183, 10–17.

Cariou, S., Catzavelos, C., Slingerland, J.M., 1998. Prognostic implications of expression of the cell cycle inhibitor protein p27Kip1. Breast Cancer Res. Treat. 52, 29–41.

Musgrove, E.A., Davison, E.A., Ormandy, C.J., 2004. Role of the CDK inhibitor p27 (Kip1) in mammary development and carcinogenesis: insights from knockout mice. J. Mammary Gland Biol. Neoplasia 9, 55–66.

Alkarain, A., Jordan, R., Slingerland, J., 2004. P27 deregulation in breast cancer: prognostic significance and implications for therapy. J. Mammary Gland Biol. Neoplasia 9, 67–80.

Tawfic, S., Yu, S., Wang, H., Faust, R., Davis, A., Ahmed, K., 2001. Protein kinase CK2 signal in neoplasia. Histol. Histopathol. 16, 573–582.

Wilson, G.R., Cramer, A., Welman, A., Knox, F., Swindell, R., Kawakatsu, H., Clarke, R.B., Dive, C., Bundred, N.J., 2006. Activated c-SRC in ductal carcinoma in situ correlates with high tumour grade, high proliferation and HER2 positivity. Br. J. Cancer 95, 1410–1414.

Dressing, G.E., Lange, C.A., 2009. Integrated actions of progesterone receptor and cell cycle machinery regulate breast cancer cell proliferation. Steroids 74, 573–576.

Narayanan, R., Adigun, A.A., Edwards, D.P., Weigel, N.L., 2005. Cyclin-dependent kinase activity is required for progesterone receptor function: novel role for cyclin A/Cdk2 as a progesterone receptor coactivator. Mol. Cell Biol. 25, 264–277.

Weigel, N.L., Moore, N.L., 2007. Cyclins, cyclin dependent kinases, and regulation of steroid receptor action. Mol. Cell Endocrinol. 265–266, 157–161.

Zhang, Y., Beck, C.A., Poletti, A., Edwards, D.P., Weigel, N.L., 1994. Identification of phosphorylation sites unique to the B form of human progesterone receptor in vitro phosphorylation by casein kinase II. J. Biol. Chem. 269, 31034–31040.

Trembley, J.H., Wang, G., Unger, G., Slaton, J., Ahmed, K., 2009. Protein kinase CK2 in health and disease: CK2: a key player in cancer biology. Cell Mol. Life Sci. 66, 1858–1867.

Beral, V., 2003. Breast cancer and hormone-replacement therapy in the million women study. Lancet 362, 419–427.

Anderson, G.L., Limacher, M., Assaf, A.R., Bassford, T., Beresford, S.A., Black, H., Bonds, D., Brunner, R., Brzyski, R., Caan, B., Chlebowski, R., Curb, D., Gass, M., Hays, J., Heiss, G., Hendrix, S., Howard, B.V., Hsia, J., Hubbell, A., Jackson, R., Johnson, K.C., Judd, H., Kotchen, J.M., Kuller, L., LaCroix, A.Z., Lane, D., Langer, R.D., Lasser, N., Lewis, C.E., Manson, J., Margolis, K., Ockene, J., O'Sullivan, M.J., Phillips, L., Prentice, R.L., Ritenbaugh, C., Robbins, J., Rossouw, J.E., Sarto, G., Stefanick, M.L., Van Horn, L., Wactawski-Wende, J., Wallace, R., Wassertheil-Smoller, S., 2004. Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. JAMA 291, 1701–1712.

Chlebowski, R.T., Hendrix, S.L., Langer, R.D., Stefanick, M.L., Gass, M., Lane, D., Rodabough, R.J., Gilligan, M.A., Cyr, M.G., Thomson, C.A., Khandekar, J., Petrovitch, H., McTiernan, A., 2003. Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: the Women's Health Initiative Randomized Trial. Jama 289, 3243–3253.

Ballare, C., Uhrig, M., Bechtold, T., Sancho, E., Di Domenico, M., Migliaccio, A., Auricchio, F., Beato, M., 2003. Two domains of the progesterone receptor interact with the estrogen receptor and are required for progesterone activation of the c-Src/Erk pathway in mammalian cells. Mol. Cell Biol. 23, 1994–2008.

Vicent, G.P., Ballare, C., Nacht, A.S., Clausell, J., Subtil-Rodriguez, A., Quiles, I., Jordan, A., Beato, M., 2006. Induction of progesterone target genes requires activation of Erk and Msk kinases and phosphorylation of histone H3. Mol. Cell. 24, 367–381.

Quiles, I., Millan-Arino, L., Subtil-Rodriguez, A., Minana, B., Spinedi, N., Ballare, C., Beato, M., Jordan, A., 2009. Mutational analysis of progesterone receptor functional domains in stable cell lines delineates sets of genes regulated by different mechanisms. Mol. Endocrinol. 23, 809–826.

Faivre, E., Skildum, A., Pierson-Mullany, L., Lange, C.A., 2005. Integration of progesterone receptor mediated rapid signaling and nuclear actions in breast cancer cell models: role of mitogen-activated protein kinases and cell cycle regulators. Steroids 70, 418–426.

Proietti, C.J., Rosemblit, C., Beguelin, W., Rivas, M.A., Diaz Flaque, M.C., Charreau, E.H., Schillaci, R., Elizalde, P.V., 2009. Activation of Stat3 by heregulin/ErbB-2 through the co-option of progesterone receptor signaling drives breast cancer growth. Mol. Cell Biol. 29, 1249–1265.

Beguelin, W., Diaz Flaque, M.C., Proietti, C.J., Cayrol, F., Rivas, M.A., Tkach, M., Rosemblit, C., Tocci, J.M., Charreau, E.H., Schillaci, R., Elizalde, P.V., 2010. Progesterone receptor induces ErbB-2 nuclear translocation to promote breast cancer growth via a novel transcriptional effect: ErbB-2 function as a coactivator of Stat3. Mol. Cell Biol. 30, 5456–5472.

Proietti, C.J., Beguelin, W., Flaque, M.C., Cayrol, F., Rivas, M.A., Tkach, M., Charreau, E.H., Schillaci, R., Elizalde, P.V., 2010. Novel role of signal transducer and activator of transcription 3 as a progesterone receptor coactivator in breast cancer. Steroids 76, 381–392.

Richer, J.K., Lange, C.A., Manning, N.G., Owen, G., Powell, R., Horwitz, K.B., 1998. Convergence of progesterone with growth factor and cytokine signaling in breast cancer. Progesterone receptors regulate signal transducers and activators of transcription expression and activity. J. Biol. Chem. 273, 31317–31326.

Cerliani, J.P., Guillardoy, T., Giulianelli, S., Vaque, J.P., Gutkind, J.S., Vanzulli, S.I., Martins, R., Zeitlin, E., Lamb, C.A. and Lanari, C., 2011. Interaction between FGFR-2, STAT5 and progesterone receptors in breast cancer, Cancer Res.

Santos, S.J., Haslam, S.Z., Conrad, S.E., 2010. Signal transducer and activator of transcription 5a mediates mammary ductal branching and proliferation in the nulliparous mouse. Endocrinology 151, 2876–2885.

Santos, S.J., Haslam, S.Z., Conrad, S.E., 2008. Estrogen and progesterone are critical regulators of Stat5a expression in the mouse mammary gland. Endocrinology 149, 329–338.

Jacobsen, B.M., Richer, J.K., Sartorius, C.A., Horwitz, K.B., 2003. Expression profiling of human breast cancers and gene regulation by progesterone receptors. J. Mammary Gland Biol. Neoplasia. 8, 257–268.

Chlebowski, R.T., Kuller, L.H., Prentice, R.L., Stefanick, M.L., Manson, J.E., Gass, M., Aragaki, A.K., Ockene, J.K., Lane, D.S., Sarto, G.E., Rajkovic, A., Schenken, R., Hendrix, S.L., Ravdin, P.M., Rohan, T.E., Yasmeen, S., Anderson, G., 2009. Breast cancer after use of estrogen plus progestin in postmenopausal women. N. Engl. J. Med. 360, 573–587.

Seagroves, T.N., Lydon, J.P., Hovey, R.C., Vonderhaar, B.K., Rosen, J.M., 2000. C/EBPbeta (CCAAT/enhancer binding protein) controls cell fate determination during mammary gland development. Mol. Endocrinol. 14, 359–368.

Osborne, C.K., Schiff, R., Arpino, G., Lee, A.S., Hilsenbeck, V.G., 2005. Endocrine responsiveness: understanding how progesterone receptor can be used to select endocrine therapy. Breast 14, 458–465.

Gregory, C.W., Fei, X., Ponguta, L.A., He, B., Bill, H.M., French, F.S., Wilson, E.M., 2004. Epidermal growth factor increases coactivation of the androgen receptor in recurrent prostate cancer. J. Biol. Chem. 279, 7119–7130.

Steeg, P.S., Zhou, Q., 1998. Cyclins and breast cancer. Breast Cancer Res. Treat 52, 17–28.

Jeong, Y., Xie, Y., Xiao, G., Behrens, C., Girard, L., Wistuba, II, Minna, J.D., Mangelsdorf, D.J., 2010. Nuclear receptor expression defines a set of prognostic biomarkers for lung cancer. PLoS Med. 7, e1000378.

Joshi, P.A., Jackson, H.W., Beristain, A.G., Di Grappa, M.A., Mote, P.A., Clarke, C.L., Stingl, J., Waterhouse, P.D., Khokha, R., 2010. Progesterone induces adult mammary stem cell expansion. Nature 465, 803–817.

Asselin-Labat, M.L., Shackleton, M., Stingl, J., Vaillant, F., Forrest, N.C., Eaves, C.J., Visvader, J.E., Lindeman, G.J., 2006. Steroid hormone receptor status of mouse mammary stem cells. J. Natl. Cancer Inst. 98, 1011–1014.

EXPERT REVIEWS

Progesterone receptor action: defining a role in breast cancer

Expert Rev. Endocrinol. Metab. 6(3), xxx–xxx (2011)

Andrea R Daniel[1], Christy R Hagan[1] and Carol A Lange[†1]

[1]*Departments of Medicine (Division of Hematology, Oncology, and Transplantation) and Pharmacology, and The Masonic Cancer Center, University of Minnesota, Minneapolis, MN 55455, USA*
[†]*Author for correspondence:*
Tel.: +1 612 626 0621
Fax: +1 612 624 3913
lange047@umn.edu

The ovarian steroid hormones, estradiol and progesterone, and their nuclear receptors (estrogen receptor [ER] and progesterone receptor [PR]), are involved in breast cancer development. As ER-positive/PR-positive tumors progress, they are likely to become steroid hormone-resistant/independent, yet often retain expression of their steroid receptors. Notably, up to 40% of women with steroid receptor-positive tumors exhibit *de novo* resistance or eventually fail on estrogen- or ER-α-blocking therapies (acquired resistance). Indeed, most of the research on this topic has centered on mechanisms of ER 'escape' from endocrine therapy and the design of better ER-blocking strategies; signaling pathways that mediate endocrine (i.e., anti-estrogen) resistance are also excellent therapeutic targets. However, serious consideration of PR isoforms as important drivers of early breast cancer progression and ER modulators is timely and significant. Indeed, progress has been hindered by ER-centric experimental approaches. This article will focus on defining a role for PR in breast cancer with hopes of providing a refreshing PR-focused perspective.

KEYWORDS: breast cancer • estrogen receptor • hormone replacement therapy • mammary gland biology • progesterone receptor • protein kinases • stem cells

Progesterone receptor isoforms are multifunctional transcription factors

Progesterone receptors (PRs) are ligand-activated transcription factor members of the steroid hormone receptor (SR) subfamily of nuclear receptors (FIGURE 1). Two common isoforms (A and B) are created from the same gene via alternate translational start sites; PR-B refers to the full-length receptor, while PR-A is an N-terminally truncated version (missing the first 164 amino acids found in PR-B). The *PR* gene is differentially regulated by two independent (isoform-specific) promoters. A and B isoforms can act as homo- (A:A or B:B) or heterodimers (A:B) and are capable of binding DNA at progesterone response elements [1] and/or via tethering to other transcription factors (signal transducers and activators of transcription [STATs], specificity protein 1 [SP1] and activator protein 1 [AP1]) [2–5]. PR-A and -B can regulate the same or different (isoform-specific) sets of target genes and exhibit both ligand-dependent and -independent activities [6,7]; these PR functions are heavily influenced by cross-talk/input from peptide growth factor-initiated signal transduction pathways [8]. A third PR isoform termed PR-C is truncated still further downstream by use of an additional AUG codon within the DNA-binding domain; this highly tissue-specific receptor inhibits the actions of PR-B in the uterus and is important for the induction of labor [9].

Steroid hormone receptors function as signal transduction molecules. PRs function not only as critical regulators of transcription but also to activate signal transduction pathways, many of which are involved in pro-proliferative signaling in the breast. Because normal (cycling) mammary epithelial cells are devoid of estrogen receptor (ER) and PR, studies on the biochemistry of PR action have largely employed ER-positive (ER+)/PR-positive (PR+) human breast cancer cell lines (MCF-7, T47D and ZR-75). Emerging *in vitro* data suggest that PR extranuclear (nongenomic) actions lead to rapid activation of protein kinases (MAPK, PI3K/Akt and c-Src) in part by a ligand-induced interaction between PR and c-Src kinase [10–12]. Seminal work from Migliaccio *et al.* demonstrated that synthetic progesterone treatment rapidly activated c-Src and ERK2 (MAPK) in breast cancer cells (T47D), and this MAPK activation translated into an increase in T47D cell growth (FIGURE 2) [10]. These data showed that c-Src activation was dependent upon an interaction between PR, c-Src and, surprisingly, ER-α; treatment with anti-estrogens blocked progesterone-induced MAPK activation. Interestingly,

 10.1586/EEM.11.25 ISSN 1744-6651

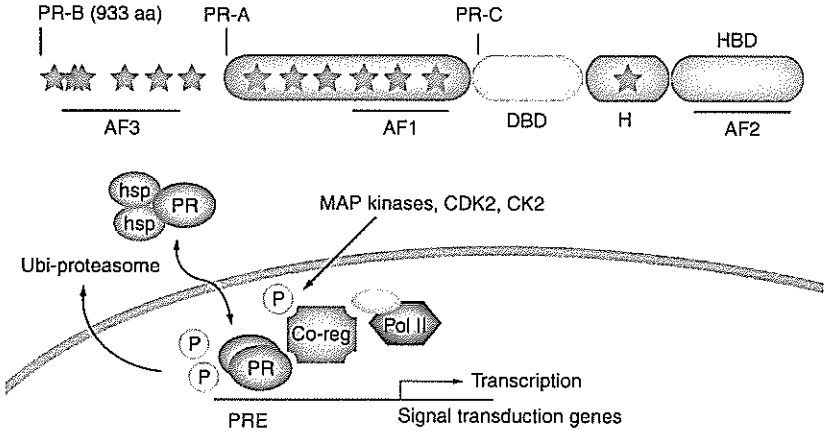

Figure 1. Progesterone receptor isoforms are sensors for growth factor-induced signaling. PR-B and truncated PR-A are substrates for mitogenic protein kinases, including CDK2 (up to eight sites, including Ser400), MAPKs (Ser294 and Ser345) and CK2 (Ser81). Phosphorylated (P) receptors and/or coregulators of transcription (such as steroid receptor coactivators) mediate promoter selection and sensitivity of PR target genes to progesterone and other hormones, including peptide growth factors (EGF, FGF receptor or IGF). Up to 14 sites (stars) in PR-B are phosphorylated either basally and/or in response to hormone action; MAPK- or CDK2-dependent phosphorylation of PR Ser294 facilitates ligand-dependent nuclear export and receptor downregulation via targeting to the ubiquitin–proteasome pathway.
AF: Activation function; DBD: DNA-binding domain; ER: Estrogen receptor; H: Hinge; HBD: Hormone-binding domain; hsp: Heat-shock protein; P: Phosphorylation; Pol II: RNA polymerase II; PR: Progesterone receptor; PRE: Progesterone response element.

in these studies, no direct interaction between PR and c-Src was observed, implicating ER as a linker molecule in heterotrimeric signaling complexes. Subsequent work from this group identified two ER-interacting domains within PR that are responsible for mediating PR/ER/c-Src interactions [13]. Complementary work from Boonyaratanakornkit *et al.* reached a similar conclusion; rapid activation of c-Src/MAPK was observed following treatment with progestins [11]. However, *in vitro*, signaling occurred independently of PR interaction with ER. These researchers identified a direct interaction between an N-terminal proline-rich region of PR and the SH3-domain of c-Src. In contrast to what was previously observed (described earlier), progestin-induced MAPK levels were low (25% of EGF-treated positive control), and did not translate to increased cell growth; this group observed a drop in progesterone-induced cell growth inhibition in PR-null normal mammary epithelial cells (MCF10A) stably expressing mutant PR-B incapable of interacting with c-Src (relative to cells expressing wild-type PR-B) [11].

The rapid signaling and transcriptional activities of PR are integrated events. Although the rapid signaling actions of SRs take place independently of transcription (i.e., in seconds to minutes), it is becoming increasingly clear that membrane-initiated and nuclear functions of SRs are fully integrated events (Figure 2). For example, Faivre *et al.* first demonstrated a mechanism of progestin/PR-induced autocrine signaling in which rapid signaling complexes (containing PR and c-Src) are required for subsequent expression of PR-target genes (including EGF receptor [*EGFR*] and *WNT1*) [14]. In response to progestins, secreted WNT1 activates frizzled receptors on the cell surface, leading to matrix metalloproteinase production and cleavage of heparin-binding EGF molecules (i.e., to produce free EGF). Progestin-dependent transactivation of EGFR ultimately induces sustained MAPK activation, cyclin D1 expression, and increased cell proliferation and survival [14]. In this model, rapid or membrane-associated PR signaling induces c-Src- and MAPK-dependent phosphorylation of PR Ser345 [15]. Phosphorylation of PR Ser345 is required for PR tethering to SP1, a transcription factor mediator of progestin-responsive genes, such as *p21* and *EGFR*. These data demonstrated that PR-containing rapid signaling complexes function to transmit specific information (i.e., in the form of phosphorylation events) to genomic transcriptional complexes. Related to this concept, intriguing new data from Béguelin *et al.* defined a novel model for PR cross-talk with signaling complexes that involved progestin-induced activation and nuclear translocation of ErbB2, a membrane-associated receptor tyrosine kinase [16]. Once localized in the nucleus, ErbB2 formed a transcriptional complex with PR and STAT3, serving as a transcriptional coactivator for STAT3 and controlling genes such as cyclin D1. Inhibiting formation of this transcriptional complex prevented progestin-driven PR/ErbB2-positive tumor growth in mouse models. Taken together, these data support a novel role for PR involving a hybrid of extranuclear and genomic actions: ligand-activated PR induces EGFR [14] or ErbB2 [16] transactivation and subsequent transcriptional complex formation, with nuclear PR being a critical component of this protein complex at selected gene promoters.

Whereas the protein complex components that are critical to support progestin-induced MAPK activation remain somewhat controversial (discussed earlier), all models tend to agree that rapid activation of MAPKs by progestins is mediated by membrane-associated PR, either directly or indirectly. Notably, SRs (ER, PR and androgen receptor) traffic to the plasma membrane, in part via heat-shock protein (hsp)27-dependent tethering, where they are reversibly palmitoylated in order to facilitate and prolong membrane location and function [17]. Work from these groups and others [12] underscored the important extranuclear role that SRs play in the rapid activation of cytoplasmic or membrane-associated protein kinases (c-Src and PI3K/Akt), and downstream signaling cascades (MAPKs). Importantly, these kinases modify regulatory sites on SRs, including ER

and PR [15], and their coregulators [18], thereby integrating both rapid signaling and genomic actions.

Like other SRs, PRs are significantly post-translationally modified by phosphorylation, acetylation, sumoylation and ubiquitination [19–23]. These modifications are often ligand dependent, but can also occur independently of ligand binding (primarily in response to protein kinase activation), and significantly alter receptor stability, localization, tethering interactions, transcriptional activity and promoter selectivity [24]. For example, MAPK and cdk2 have previously been demonstrated to phosphorylate and modulate the activity of both liganded and unliganded PR [21,25–27]. Phospho-PRs are targeted to specific gene subsets, and subsequent specific transcriptional profiles depend on the phosphorylation status of PR [15,19,28]. Thus, a feed-forward loop between progestin-activated protein kinases and subsequent phosphorylation of PRs (by those same kinases) underlies the profound effects that activated kinases have on the nuclear functions of PR, particularly with regard to promoter selectivity [14,15,28]. With the exception of K303A ER-α, a hyperactive mutant ER found in a subset of human breast cancers [29], one reason that ER and PR are seldom mutated is because these receptors are subject to intense epigenetic regulation (i.e., phosphorylation most often translates to gain of function) by the same protein kinases that are most often upregulated or constitutively activated in breast cancer. Because a myriad of post-translational inputs are capable of driving receptor and/or coregulator behaviors, there may be little pressure for adaptive mutations that accomplish that same task (however, the receptors are frequently overexpressed).

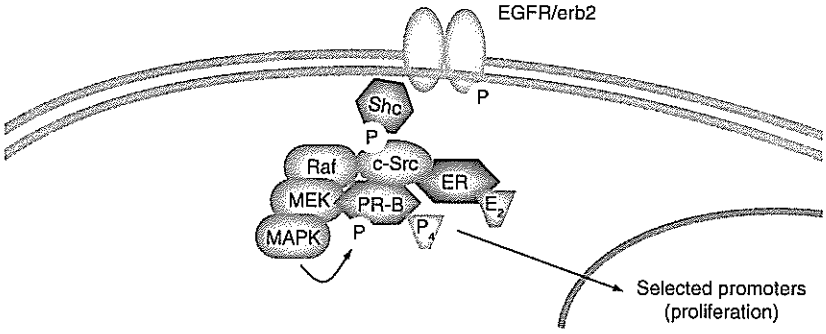

Figure 2. Progesterone receptor-B, but not progesterone receptor-A, and estrogen receptor-α participate in membrane-tethered protein complexes capable of rapidly activating c-Src and MAPKs. Progesterone/PR and estrogen/ER transactivate EGFR and/or ErbB2, phosphorylated (P) steroid hormone receptors and signaling molecules, including protein kinases and surface receptors, enter the nucleus and participate in transcription complexes at selected gene promoters.
E2: Estradiol; EGFR: EGF receptor; ER: Estrogen receptor; PR: Progesterone receptor; Shc: Src homology domain II containing.

Growth factor- or SR-induced rapid signaling provides a mechanism for PR promoter selection. These types of data underscore the concept that so-called rapid signaling actions of SRs simply constitute a required step in the pathway to gene regulation and, specifically, promoter selection (i.e., by the very same receptors). That is, rapid and dynamic shuttling of SRs between the cytoplasmic and nuclear compartments allows for constant interaction with protein kinases; SRs are in fact sensors for the actions of growth factors and signaling molecules stationed within and at the plasma membrane. Thus, although extranuclear PR actions are often considered to be functionally distinct from downstream genomic PR events (they are most often studied separately), cytoplasmic and nuclear receptors are probably part of the same dynamic or 'fluxing' population (Figure 2). In response to hormonal cues, cycling populations of transiently membrane-localized PRs rapidly activate appropriate protein kinase cascades. These kinases phosphorylate nearby substrates (i.e., membrane-tethered PRs and cytoplasmic coregulators). Entire complexes containing steroid receptor phosphospecies, coregulators and signaling molecules (including kinases) then associate dynamically with regulatory regions/enhancers in DNA to activate or inhibit gene expression. This scheme explains why some SR-dependent promoters are exquisitely sensitive to alterations in protein kinase activities (a minority of receptors are membrane associated at any given time), while others are much more tightly regulated by steroid hormone alone [15]. Overall, kinase signaling (including SR-dependent rapid signaling) is a mechanism for promoter selection; it provides a means of quickly altering hormone responsiveness at some, but not all, promoters. This is an important facet of PR action and explains why PR gene signatures differ in normal versus neoplastic mammary epithelial cells [30]; under the influence of signal transduction pathways commonly activated in breast cancer cells, PR signaling and thus promoter selection, differs dramatically, resulting in altered cell/tumor biology.

Progesterone is a potent breast mitogen. Once a controversial notion, it is now well accepted that progesterone acts as a proliferative hormone in the breast, although it is paradoxically inhibitory in the reproductive tract and ovaries. A primary function of progesterone/PR is to mediate the massive expansion of epithelial-derived mammary alveoli (alveologenesis and organization of alveoli into lobules) during puberty and pregnancy in preparation for lactation. Increased serum levels of progesterone during the luteal phase of the menstrual cycle are coincident with a high proliferative index of epithelial cells in the milk duct system [31]. Likewise, during diestrus in mice, when progesterone levels rise by approximately fourfold, an increase in ductal structures is visible in mammary gland whole mounts [32]. Mouse knockout studies demonstrated that PR-B, rather than PR-A, is specifically required for the epithelial cell proliferation that is the basis of extensive mammary gland ductal side branching and alveologenesis [33]. Studies in receptor activator of NF-κB ligand (RANKL) and cyclin D1 (i.e., both major downstream effectors of PR)-deficient mice show similar blocks in alveologenesis [34,35],

while receptor of activator of NF-κB (RANK)-transgenic mice express increased cyclin D1 and undergo increased hormone-driven proliferation and mammary tumor formation [36]. In contrast to PR-B, ER-α is required for mammary ductal elongation prior to pregnancy when the gland is highly responsive to estrogen, but relatively unresponsive to progesterone [37]. Estrogen/ER also contributes to alveolar development, in part via induction of PR expression [38].

Steroid receptor action is required for normal mammary gland development. Like ER-α, PR isoforms are found in a minority of mammary epithelial cells (MECs). These receptors are most often coexpressed, occurring in only approximately 10–20% of luminal epithelial cells in the normal mammary gland [37]. Multiple studies have concluded that SR-negative (SR-) cells comprise the majority of the proliferating (nonpregnant) normal MEC cohort [39–41]. Thus, in response to progesterone, it has been proposed that PR+ cells provide mitogenic paracrine signals that direct neighboring SR-negative cells to divide (Figure 3) [42]. Recently, Beleut *et al.* described two distinct mechanisms of progesterone-induced MEC proliferation that occurred in waves following progesterone administration to adult ovariectomized mice [43]. Initially peaking approximately 24 h post-treatment, a subset of PR+ cells (5% of MECs) in the luminal compartment were stimulated to divide. Cyclin D1, a PR target gene, was required for this cell-autonomous proliferative response. After approximately 3 days of progesterone treatment, a second wave of proliferation peaks (27% of MECs); this fraction of cells is PR null but dependent upon the PR-induced paracrine factor, RANKL, for mitogenic stimulation (Figure 3). Similarly, WNT4, another paracrine mitogen induced by PR, is required for progesterone-induced side-branching during the development of mammary ducts [44]. Other studies performed in mice and rats also illustrate that a small percentage of PR-B, but not PR-A, expressing MECs actively undergo cell division, as measured by BrdU incorporation and PR co-staining; proliferation of PR-B containing cells becomes extensive during pregnancy [45]. Regulation of PR isoform expression is poorly understood in humans. However, in rodent models, estrogen induced PR-A expression, while progesterone alone or estrogen plus progesterone were required for significant PR-B expression [37]. In summary, in the normal breast, estrogen/ER may primarily act to increase PR-A expression [37], while progesterone/PR-B initiates a series of potent proliferative factors (WNT4, cyclin D1 and RANKL) for exquisitely timed expansion of the mammary gland.

Hormone-dependent breast cancers undergo an early switch to autocrine growth signaling. Despite the relatively low abundance of MECs in the normal (i.e., nonpregnant) breast that express SRs, the majority of breast cancers are ER+/PR+ upon initial diagnosis [46]. Numerous models, both *in vitro* and *in vivo*, demonstrate that progesterone/PR remains a strong mitogenic and prosurvival stimulus within the context of breast cancer [8]. PR, in the presence and absence of ligand, induced anchorage-independent growth and increased survival in breast cancer cell lines [14,28,47]. In mouse models, mammary tumors induced by chemical carcinogens and genetic disruption of the tumor suppressor, BRCA1, were dependent on PR action [48,49]. In addition, administration of medroxyprogesterone acetate induced mammary carcinogenesis in multiple species, including mice [50]. Furthermore, in rats, CDB-4124, a clinically used (for uterine fibroids and endometriosis) antiprogestin/PR modulator (PRM), inhibited the appearance of spontaneous preneoplastic mammary lesions and *N*-methyl-*N*-nitrosourea-induced (ER+) mammary tumors, primarily via suppression of proliferation and induction of apoptosis [51]. A few small clinical trials have used additional PRMs to target PR in breast cancer with good success, despite cross-reactivity with glucocorticoid receptors [52,53]. Finally, large clinical trials have demonstrated that progestin added to hormone replacement therapy significantly increased the incidence and grade of breast tumors in postmenopausal women [54]. No increased risk was associated with estrogen alone [54,55], and estrogen-only hormone replacement therapy may be protective in some women.

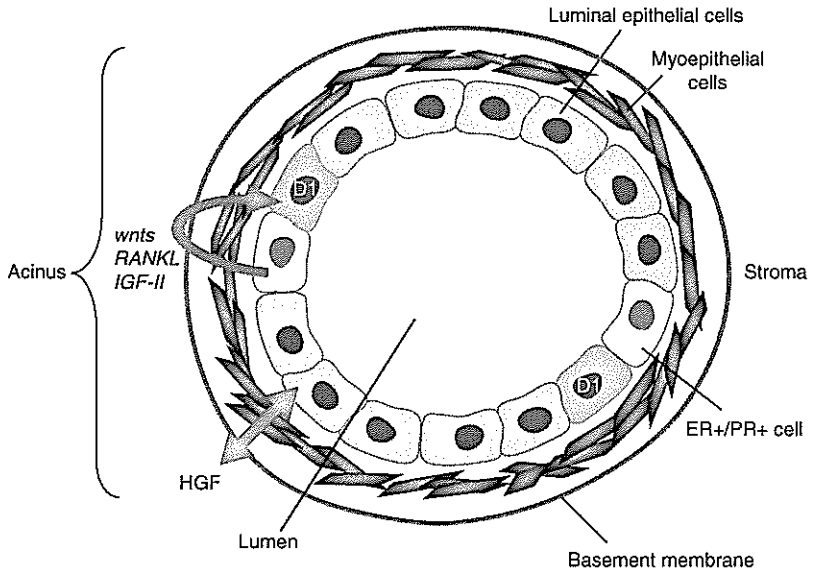

Figure 3. Proliferating cells in the normal (non-pregnant) mammary gland are typically steroid hormone receptor null. ER and PR isoforms are co-expressed in a minority population of mammary epithelial cells that lie adjacent to proliferating (cyclin D1-positive) SR-negative cells. Progesterone/PR-dependent paracrine factors (WNTs, RANKL and IGF-II) induce neighboring (PR-null) cells to undergo proliferation. An early switch to autocrine signaling mechanisms occurs in development of ER+/PR+ breast cancers.
ER: Estrogen receptor; PR: Progesterone receptor.

Synthetic progestins used in hormone replacement therapy clinical trials and progesterone have overlapping effects on PR [50]; therefore, progesterone is not considered an entirely safe alternative.

Interestingly, gene-expression analysis of normal human MECs cultured in 3D relative to similarly cultured T47D human breast cancer cells showed distinct genetic profiles upon progestin treatment, indicating that progesterone-induced proliferative programs differ between normal and cancer cells [30]. This is not entirely surprising, considering that in the normal (nonpregnant) breast, the majority of proliferating cells are devoid of SRs and instead primarily divide in response to paracrine signals; in SR-positive breast tumors, PR-containing cells proliferate, presumably via autocrine mechanisms that may be Wnt1-, EGFR- and cyclin D1-dependent [14]. In addition, mitogenic protein kinases (CDK2, c-Src, CK2 and MAPK), often upregulated in breast cancer, drive PR hypersensitivity to ligand and ligand-independent activity, and can also redirect phospho-PR to alternate promoters ([10,15,19,28,56]; discussed further later).

Progesterone mediates mammary gland stem cell self-renewal. Lifetime exposure to steroid hormones (either exogenous or endogenous) is a critical risk factor for the development of breast cancer. For example, a greater number of menstrual cycles (experienced over an individual's lifetime) is correlated with increased breast cancer incidence [57]. Accumulating evidence implicates progesterone/PR in the maintenance and expansion of breast stem and progenitor cells. It has been proposed that mammary stem cells (MaSCs) comprise a population of putative primary targets for transformation to breast malignancies [58,59]. Quiescent MaSCs are thought to be activated during periods of glandular expansion, such as puberty and pregnancy [59–61], when progesterone levels are high. Early reports described hormone receptor-positive (30–40%) and -negative cells that divide asymmetrically (as measured by DNA labeling) in mice undergoing puberty, and proliferate again in adulthood upon hormone administration [61–63]. Others reported mouse MaSCs to be ER-/PR- cells surrounded by myoepithelial and luminal cells, some of which express both ER and PR [64]. Similarly, in humans, the cell populations enriched for MaSCs have been reported to be both SR+ [65] and SR- [66]. It is likely that MaSCs are SR-, yet require local SR+ cells to provide paracrine signals [58]. Shackelton *et al.* were able to generate functional mammary glands from MaSCs isolated from a niche in the basal epithelial layer [60].

Recently, progesterone was shown to induce basal MaSC ($CD49f^{hi}$) expansion in the diestrus phase of cycling female mice [32]. The authors suggest that PR induction of WNT4 and RANKL in the luminal compartment act in a paracrine manner to enrich the basal MaSC population. Genetically engineered mice with RANK deleted from mammary epithelial cells were resistant to progestin-induced epithelial proliferation and expansion of $CD49^{hi}$ stem cells; these mice also exhibited sensitization to DNA-damaging agents [67]. While these are intriguing results, the contribution of RANK to human breast development and cancer awaits confirmation [68]. In primary human breast cultures, Graham *et al.* described an increase in progenitor cell populations in response to progesterone treatment [30]. Recent work in human MECs showed that WNT1, a progesterone-regulated gene [14], is upstream of Notch signaling [69], which is implicated in affecting stem cell self-renewal and lineage-specific differentiation in the mammary gland [70]. It is thus reasonable to predict that progesterone may also drive the expansion of breast cancer progenitor cells, a hypothesis examined by Horwitz *et al.* [71,72]. In these studies, T47D human breast cancer cell xenografts were reported to contain a rare population of basal-like $CD44^+$ tumor-initiating cells ($ER^-PR^-CK5^+$), an intermediate cell population ($ER^-PR^-CK5^-$) and an expanding population of luminal-like cells ($ER^+PR^+CK5^-$). Upon treatment with progestin, $ER^+PR^+CK5^+$ cells were observed and $ER^-PR^-CK5^+$ cells were enriched. The authors propose that the $ER^+PR^+CK5^+$ cells comprise a transitional cell population present in tumors that may retrogress to $ER^-PR^-CK5^+$ cells in response to progestins [71,72]. As a result, progesterone maintenance and expansion of MaSCs may have implications for breast tumor stem cell populations; these cells are likely to be more resistant to traditional cancer therapies due to their ability to undergo quiescence, a state characterized by a high degree of resistance to apoptosis and agents that primarily target properties of rapidly dividing cells (i.e., classical chemotherapies). Going forward, it will be critical to delineate important similarities and differences between the various models used to study these hormone-dependent aspects of mammary gland biology; significant differences exist between mice (the primary genetic model employed in breast cancer research), humans and rats. The inclusion of more rat models may provide further insight into steroid receptor biology in mammary gland development and tumor progression (reviewed in [37]).

Expert commentary

Is the action of progesterone receptors in breast cancer a missed opportunity? Owing to a convergence of factors, PR action in breast cancer has been almost entirely overlooked. First, the topic is complex. The natural hormone, progesterone, has opposing effects according to target tissue and cell context. Progestins are mitogenic in the breast, but inhibitory in the uterus and ovaries; the basis for this divergence is still unknown. Human breast cancer cells cultured in 2D (plastic dishes) exhibit a biphasic pattern of growth in response to progesterone (when subjected to continuous progesterone treatment, they undergo one or more rounds of cell division and are then growth inhibited [73]). In addition, genetically engineered mice are the primary animal models used in breast cancer research. As both ER and PR are required for mammary gland development, interpretation of studies using ER or PR-knockout mice are limited in that these animals lack the structures/cells that give rise to breast cancer (i.e., mammary gland development is severely impaired). Unlike the human breast, the mouse mammary gland does not fully develop until pregnancy; virgin glands are relatively unresponsive to progesterone and primarily express PR-A, but contain very little PR-B. Indeed, few genetic mouse models develop ER+/PR+ mammary tumors [74]. Furthermore, studies of human breast oncogenes (i.e., transgenic mouse models) frequently evaluate virgin animals, making it impossible to implicate PR-B (i.e., the proliferative receptor) in tumor biology. This may partly explain why treatment of well-established animal tumor models

with progestin (agonists) rarely augments tumor biology (although the use of antiprogestin [antagonists] is often inhibitory; discussed later). Antiprogestins were rejected in early human clinical trials not because they were not highly effective [53], but because they had significant cross-reactivity with their glucocorticoid receptor close cousins, resulting in intolerable side effects (reviewed in [75]). Finally, considerable political resistance has discouraged mainstream use of antiprogestins within the USA for any indication (i.e., the antiprogestin, RU486, is clinically known as 'the abortion pill'); drug companies avoid the development of agents perceived to be unpopular or not sufficiently lucrative/patentable. For these unfortunate reasons (few of which are relevant to peer-reviewed science on this topic), PR isoforms are grossly understudied relative to ER-α in the breast and breast cancer. In fact, experts suggest that PR is a highly relevant SR with respect to both normal and neoplastic breast epithelial cell proliferation [30], early breast cancer progression [51,76,77] and, more recently, mammary gland stem-cell biology [32]. Like ER, PR mutations are not commonly seen in the majority of breast cancers, although the normal 1:1 ratio of PR-A to PR-B is frequently altered [78]; the significance of this finding is unknown but probably relates to altered homeostasis and rapidly changing patterns of gene expression during early tumor development [30].

Why study progesterone/PR in the breast? ER is the first example and the primary focus of very successful 'targeted' breast cancer therapies. However, the actions of ER and PR are intimately linked in biology. PR is an important ER target gene and thus acts as a major downstream effector of estrogen action. As mentioned previously, historically, progesterone was assumed to have little to no effect on breast tumorigenesis, partly owing to its well-established inhibitory and differentiative role in the uterus and reproductive organs. However, more recently, progesterone has been implicated as a proliferative hormone in the normal breast [30] and a lifelong risk factor for breast cancer [55,79–84]. Notably, as with ER, there is extensive cross-talk between PR and the same signal transduction pathways that are required for mammary gland development and are most often elevated in breast cancer. For example, the proliferative effects of progesterone are highly dependent upon tyrosine kinase growth factor receptors (EGFR family members) and their downstream protein kinase effectors (c-Src and MAPKs); these effects (i.e., cell proliferation) map to direct phosphorylation of PR-B, but not PR-A [14]. Cross-talk between PR-B and the EGFR pathway provides a basis for understanding mechanisms of transcriptional synergy between progestins and EGF on numerous endogenous genes that are highly relevant to breast cancer biology [85]. PR target genes such as WNTs [14,44] are secreted factors that may contribute to paracrine and autocrine proliferation signals during progression to malignant transformation [69]. The physiological significance of EGF-induced PR-B hyperactivation relates to the key role of both molecules, along with ER-α, as mediators of massive alveolar proliferation during mammary gland development/early pregnancy [86]. This interplay between growth factors and both SR (ER/PR) functions (inappropriately) during breast cancer progression, when tyrosine kinase activities are elevated and hyperactive SRs are still present and functional (although frequently at low abundance; discussed further later). For this reason, targeted therapies against ER and ErbB (EGFR/ErbB2) family members are now a clinical mainstay, but their success can be limited by mechanisms of tumor progression. The addition of PR-blocking therapies to this list could be life saving; antiprogestins are predicted to severely impair the process of tumor progression (i.e., by blocking PR-induced upregulation of signaling pathway intermediates that include known mediators of endocrine resistance), which invariably occurs upon exposure to anti-estrogens or estrogen blockers [87–89]. Indeed, this is a missed opportunity for women facing fewer and fewer treatment options as they fail classical endocrine therapies.

More abundant PR may not translate to increased transcriptional activity. An early event in tumor development includes an altered ratio of coexpressed PR-A to PR-B (normally observed to be 1:1), with loss of PR-B (i.e., apparent predominance of PR-A) occurring most often [78,90]. The natural assumption is that PR-A is thus the dominant isoform, perhaps even driving tumor phenotype. However, it is also well appreciated that liganded SRs are rapidly downregulated relative to their inactive forms. Thus, the expression of phosphorylated receptors (namely PR-B) may appear to be low in PR-driven tumors due to increased phospho-PR ubiquitinylation and rapid protein 'loss' by proteasome-mediated turnover of activated receptors [21]; growth factors also ultimately lower PR mRNA expression via reversible mechanisms [21,91,92]. SR proteins and their coregulators are direct targets of growth factor-activated cytoplasmic protein kinases. Thus, a 'vicious cycle' is created, wherein growth factors induce phosphorylated and transcriptionally hyperactive PRs that turn over even more rapidly, making low-abundance receptors nearly 'invisible' at the protein level. However, their robust nuclear activity is clearly detected in reporter gene assays and at endogenous genes using subphysiologic hormone concentrations [19,21,85]. In fact, apparent 'loss' of PR is an excellent clinical marker of high growth factor receptor expression and activity [92]. This high-kinase condition is responsible for phosphorylating PR and increasing both its transcriptional activity and rate of turnover. Hyperactive PR protein may be relatively undetectable by clinically employed antibody-binding assays; when protein levels are measured, clinicians may mistakenly conclude that apparently 'PR-null' tumors have escaped hormonal regulation. Instead, 'loss' of PR-B may in fact be an excellent early marker of PR-B-driven biology; similar mechanisms have been reported for ER in breast cancer cells containing activated c-Src kinase [93]. Importantly, we reported that hyperactive (deSUMOylated) phospho-PR-B is capable of driving breast cancer cell proliferation and survival via the transcriptional regulation of novel PR target genes that are not known to be particularly responsive to progestin alone, but are very responsive to high kinase activities [94]. Surprisingly, these genes include novel phospho-PR-regulated genes and ER-regulated genes. Because hyperactive phospho-PR-B is largely deSUMOylated [19], it also fails to transrepress ER [23]; we suspect that the two receptors (PR and ER) cooperate at many of the same genes.

The development of the ER+/PR-null tumor phenotype may be PR driven. There is considerable functional overlap between ER and PR. Notably, many ER-regulated genes are also PR regulated

(including c-myc, cyclin D1, c-fos, STATs and IGF pathway components), and these receptors even tether to the same transcription factors (AP1 and SP1) to regulate non-classical target genes (which contain no hormone-responsive element). ER-α and PR-B also participate in similar membrane-associated, cytoplasmic (or 'rapid') signaling complexes (discussed previously) in association with EGFR and c-Src kinase upstream of the ERK1/2 MAPK module [10]. ER-α or PR-B localized near the cell membrane are both capable of transactivating EGFR [14,95]. In fact, steroid hormone-induced rapid activation of MAPK appears to be most robust when both ER-α and PR-B are coexpressed in model cell lines [10,13]. The end point of MAPK signaling is most often the regulation of nuclear transcription factors. Indeed, ER and PR are direct targets (substrates) of mitogenic protein kinases, including MAPK. This cross-talk even extends to the regulation of ER/PR interactions (Figure 4). In response to progesterone binding, SUMOylated PR isoforms (both A and B) transrepress ER [23]; MAPK-dependent phosphorylation events (namely PR Ser294) lift this repression by blocking PR SUMOylation [19]. ER and PR are most often co-expressed in early-stage breast cancer. Loss of PR mRNA and protein can indicate a functional loss of ER (ER+/PR-low or -null); this is a common assumption. However, an alternative pathway exists in which phospho-PR is under-SUMOylated and thus no longer able to transrepress ER (Figure 4). Phospho-PR instead behaves as a hyperactive or constitutive (i.e., ligand-independent) transcription factor at selected gene promoters, including those classically regulated by ER [28].

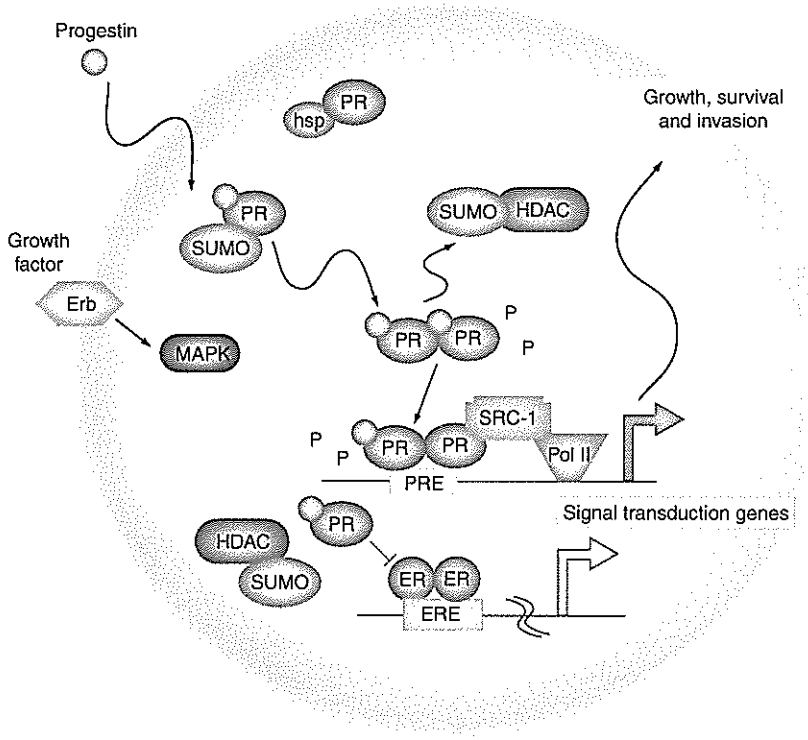

Figure 4. Reversible progesterone receptor Ser388 SUMOylation provides a mechanism for rapid changes in hormone responsiveness according to extracellular cues. PRs are rapidly SUMOylated in response to progesterone binding. SUMOylated PR species are tenfold less active on selected gene promoters and capable of ER transrepression (by unknown mechanisms). Growth factor-induced MAPK activation leading to phosphorylation of PR Ser294 prevents PR Ser388 SUMOylation, thereby lifting SUMO-dependent repression of both PR and ER transcriptional activities. Phosphorylated (P) and deSUMOylated PR-B drives breast cancer cell proliferation and survival.
ER: Estrogen receptor; Erb: Erythroblastic leukemia viral oncogene homolog; ERE: Estrogen response element; HDAC: Histone deacetylase; hsp: Heat-shock protein; Pol II: RNA polymerase II; PR: Progesterone receptor; PRE: Progesterone response element; SRC: Steroid receptor coactivator.

Five-year view

Future studies should focus on the goal of defining the contribution of protein kinase inputs to PR-dependent signaling and PR/ER cross-talk in breast cancers that are classically believed to be 'ER driven' but are resistant to anti-estrogen therapy (and may in fact be PR driven). SR-specific gene signatures, rather than protein levels (often limited to a small sampling of the tumor), should be used clinically to assess hormone responsiveness. With the development of more selective antiprogestins [51], the opportunity to understand and target the ER+/PR 'loss' phenotype as a means of combating early progression to hormone-refractory breast cancer is within reach; this phenotype can be clearly defined by the presence of a phospho-PR-B gene signature, predicted to be a sensitive and reliable readout of PR activity when PR protein levels appear to be reduced. Related to this idea, we recently defined a phospho-PR gene signature that includes both ligand-dependent and -independent PR-regulated genes; our signature predicts a high likelihood of rapid progression to breast cancer metastasis [Knutson T, Lange C, Unpublished Data]. It will now be important to validate this exciting finding in preclinical models of human breast cancer.

A wealth of basic and clinical studies have implicated PR action in breast cancer. However, only a fraction of information is known compared with that about ER, which was the first example of highly successful targeted therapy. A few tenants of PR action have emerged: PRs behave quite differently with regard to isoform specificity and cellular context (i.e., breast vs uterus or normal vs neoplastic cells); altered PR behavior is in large part conferred by the actions of activated protein kinases; PR hypersensitivity that approaches ligand independence is driven by phosphorylation events and may be significant in certain contexts; and phospho-PR may

precede/mark the near complete loss of PR protein and later growth factor-driven suppression of PR mRNA that occurs during the development and progression of endocrine-resistant luminal B-type (ER+/PR-) breast cancers. Indeed, the most appropriate use for PRMs may be during early breast cancer development or very early tumor progression (i.e., before PR levels drop precipitously). There is an increasingly recognized need to prevent or reverse the development of early lesions (i.e., that may or may not ever progress); this is a largely untapped area that warrants intense scrutiny of PRs as potentially important drivers of an early switch from SR-dependent paracrine to autocrine signaling mechanisms. The ultimate degree of aggressiveness of progressing tumors may be determined early on, partly dictated by the influence of progesterone/PR on the stem cell compartment. An increased understanding of PR function and cross-talk with ER in normal, pre-neoplastic and neoplastic settings, as well as stronger advocacy from scientist-, clinician- and patient/survivor-led groups are needed to overcome remaining resistance to the goal of including PR-targeted strategies as part of the repertory of mainstream endocrine/ER-based therapies.

Financial & competing interests disclosure

This work was supported by a grant from the National Institutes of Health (NIH/NCI): R01 CA123763 (formerly R01 DK53825; NIH/NIDDK). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Key issues

- While mouse models have significantly expanded our knowledge of breast disease, through the development and utilization of rat models we may achieve a more balanced understanding of steroid receptor regulation in breast cancer. Such models provide insight into the complex hormone-driven mechanisms of human breast cancer development and early progression, which represents a significant gap in our knowledge.
- Clinicians need to consider progesterone receptor (PR)-A and -B isoform-specific expression and action in human tumors (rather than total PR levels). Assay of well-characterized phosphorylated residues on both estrogen receptor (ER) and PR may predict clinical outcome more accurately; incorporation of steroid receptor-specific gene signatures as indicators of transcriptional activity and thus steroid receptor-driven biology is timely and feasible, and may provide the ultimate readout of endocrine status.
- Important cross-talk between growth factors and PR and between both PR isoforms and ER exists (and is the subject of highly valuable targeted therapies); PR action has been widely overlooked in this scheme. Scientists and clinicians need to work together on the development of preclinical models that clearly evaluate PR action and PR cross-talk with ER, with the goal of advancing towards routine use of PR-targeted therapies as a significant and life-saving improvement to classical endocrine therapy.

References

Papers of special note have been highlighted as:
- • of interest
- •• of considerable interest

1 Tsai SY, Carlstedt-Duke J, Weigel NL *et al.* Molecular interactions of steroid hormone receptor with its enhancer element: evidence for receptor dimer formation. *Cell* 55(2), 361–369 (1988).

2 Richer JK, Lange CA, Manning NG *et al.* Convergence of progesterone with growth factor and cytokine signaling in breast cancer. Progesterone receptors regulate signal transducers and activators of transcription expression and activity. *J. Biol. Chem.* 273(47), 31317–31326 (1998).

3 Owen GI, Richer JK, Tung L, Takimoto G, Horwitz KB. Progesterone regulates transcription of the p21(WAF1) cyclin-dependent kinase inhibitor gene through Sp1 and CBP/p300. *J. Biol. Chem.* 273(17), 10696–10701 (1998).

4 Proietti C, Salatino M, Rosemblit C *et al.* Progestins induce transcriptional activation of signal transducer and activator of transcription 3 (Stat3) via a Jak- and Src-dependent mechanism in breast cancer cells. *Mol. Cell Biol.* 25(12), 4826–4840 (2005).

5 Tseng L, Tang M, Wang Z, Mazella J. Progesterone receptor (hPR) upregulates the fibronectin promoter activity in human decidual fibroblasts. *DNA Cell Biol.* 22(10), 633–640 (2003).

6 Jacobsen BM, Richer JK, Sartorius CA, Horwitz KB. Expression profiling of human breast cancers and gene regulation by progesterone receptors. *J. Mammary Gland Biol. Neoplasia* 8(3), 257–268 (2003).

7 Richer JK, Jacobsen BM, Manning NG *et al.* Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells. *J. Biol. Chem.* 277(7), 5209–5218 (2002).

8 Lange CA, Sartorius CA, Abdel-Hafiz H *et al.* Progesterone receptor action: translating studies in breast cancer models to clinical insights. *Adv. Exp. Med. Biol.* 630, 94–111 (2008).

9 Condon JC, Hardy DB, Kovaric K, Mendelson CR. Up-regulation of the progesterone receptor (PR)-C isoform in laboring myometrium by activation of nuclear factor-κB may contribute to the onset of labor through inhibition of PR function. *Mol. Endocrinol.* 20(4), 764–775 (2006).

10 Migliaccio A, Piccolo D, Castoria G *et al.* Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. *EMBO J.* 17(7), 2008–2018 (1998).

11 Boonyaratanakornkit V, Scott MP, Ribon V *et al.* Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases. *Mol. Cell* 8(2), 269–280 (2001).

12 Saitoh M, Ohmichi M, Takahashi K *et al.* Medroxyprogesterone acetate induces cell proliferation through up-regulation of cyclin D1 expression via phosphatidylinositol 3-kinase/Akt/nuclear factor-κB cascade in human breast cancer cells. *Endocrinology* 146(11), 4917–4925 (2005).

13 Ballare C, Uhrig M, Bechtold T *et al.* Two domains of the progesterone receptor interact with the estrogen receptor and are required for progesterone activation of the c-Src/Erk pathway in mammalian cells. *Mol. Cell Biol.* 23(6), 1994–2008 (2003).

14 Faivre EJ, Lange CA. Progesterone receptors upregulate Wnt-1 to induce epidermal growth factor receptor transactivation and c-Src-dependent sustained activation of Erk1/2 mitogen-activated protein kinase in breast cancer cells. *Mol. Cell Biol.* 27(2), 466–480 (2007).

• **Biochemical characterization of progesterone receptor (PR)-dependent autocrine proliferative signaling, and provides novel evidence of the integration of rapid and genomic PR signaling.**

15 Faivre EJ, Daniel AR, Hillard CJ, Lange CA. Progesterone receptor rapid signaling mediates serine 345 phosphorylation and tethering to specificity protein 1 transcription factors. *Mol. Endocrinol.* 22(4), 823–837 (2008).

16 Béguelin W, Diaz Flaque MC, Proietti CJ *et al.* Progesterone receptor induces ErbB-2 nuclear translocation to promote breast cancer growth via a novel transcriptional effect: ErbB-2 function as a coactivator of Stat3. *Mol. Cell Biol.* 30(23), 5456–5472 (2010).

17 Razandi M, Pedram A, Levin ER. Heat shock protein 27 is required for sex steroid receptor trafficking to and functioning at the plasma membrane. *Mol. Cell Biol.* 30(13), 3249–3261 (2010).

18 Narayanan R, Adigun AA, Edwards DP, Weigel NL. Cyclin-dependent kinase activity is required for progesterone receptor function: novel role for cyclin A/Cdk2 as a progesterone receptor coactivator. *Mol. Cell Biol.* 25(1), 264–277 (2005).

19 Daniel AR, Faivre EJ, Lange CA. Phosphorylation-dependent antagonism of sumoylation derepresses progesterone receptor action in breast cancer cells. *Mol. Endocrinol.* 21(12), 2890–2906 (2007).

20 Daniel AR, Gaviglio AL, Czaplicki LM *et al.* The progesterone receptor hinge region regulates the kinetics of transcriptional responses through acetylation, phosphorylation, and nuclear retention. *Mol. Endocrinol.* 24(11), 2126–2138 (2010).

21 Lange CA, Shen T, Horwitz KB. Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome. *Proc. Natl Acad. Sci. USA* 97(3), 1032–1037 (2000).

22 Weigel NL, Bai W, Zhang Y *et al.* Phosphorylation and progesterone receptor function. *J. Steroid Biochem. Mol. Biol.* 53(1–6), 509–514 (1995).

23 Abdel-Hafiz H, Takimoto GS, Tung L, Horwitz KB. The inhibitory function in human progesterone receptor N termini binds SUMO-1 protein to regulate autoinhibition and transrepression. *J. Biol. Chem.* 277(37), 33950–33956 (2002).

24 Ward RD, Weigel NL. Steroid receptor phosphorylation: assigning function to site-specific phosphorylation. *Biofactors* 35(6), 528–536 (2009).

25 Zhang Y, Beck CA, Poletti A *et al.* Phosphorylation of human progesterone receptor by cyclin-dependent kinase 2 on three sites that are authentic basal phosphorylation sites *in vivo*. *Mol. Endocrinol.* 11(6), 823–832 (1997).

26 Pierson-Mullany LK, Lange CA. Phosphorylation of progesterone receptor serine 400 mediates ligand-independent transcriptional activity in response to activation of cyclin-dependent protein kinase 2. *Mol. Cell. Biol.* 24(24) 10542–10557 (2004).

27 Knotts TA, Orkiszewski RS, Cook RG, Edwards DP, Weigel NL. Identification of a phosphorylation site in the hinge region of the human progesterone receptor and additional amino-terminal phosphorylation sites. *J. Biol. Chem.* 276(11), 8475–8483 (2001).

28 Daniel AR, Lange CA. Protein kinases mediate ligand-independent derepression of sumoylated progesterone receptors in breast cancer cells. *Proc. Natl Acad. Sci. USA* 106(34), 14287–14292 (2009).

• **Describes a mechanism whereby kinase signals activate phospho-PR transcriptional activity in the absence of ligand to upregulate growth-promoting genes.**

29 Cui Y, Zhang M, Pestell R *et al.* Phosphorylation of estrogen receptor α blocks its acetylation and regulates estrogen sensitivity. *Cancer Res.* 64(24), 9199–9208 (2004).

30 Graham JD, Mote PA, Salagame U *et al.* DNA replication licensing and progenitor numbers are increased by progesterone in normal human breast. *Endocrinology* 150(7), 3318–3326 (2009).

• **Demonstrates that progesterone increases mammary epithelial cell (MEC) proliferation and increases progenitor cell populations using a normal human breast primary cell culture model. In addition, PR gene signatures differed between similarly cultured normal and cancer cells.**

31 Graham JD, Clarke CL. Physiological action of progesterone in target tissues. *Endocr. Rev.* 18(4), 502–519 (1997).

32 Joshi PA, Jackson HW, Beristain AG *et al.* Progesterone induces adult mammary stem cell expansion. *Nature* 465(7299), 803–807 (2010).

•• **Demonstrates that progesterone/PR is required for mammary stem cell proliferation.**

33 Conneely OM, Mulac-Jericevic B, Lydon JP, De Mayo FJ. Reproductive functions of the progesterone receptor isoforms: lessons from knock-out mice. *Mol. Cell Endocrinol.* 1(1–2), 97–103 (2001).

34 Fata JE, Kong YY, Li J *et al.* The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development. *Cell* 103(1), 41–50 (2000).

35 Sicinski P, Donaher JL, Parker SB *et al.* Cyclin D1 provides a link between development and oncogenesis in the retina and breast. *Cell* 82(4), 621–630 (1995).

36 Gonzalez-Suarez E, Jacob AP, Jones J *et al.* RANK ligand mediates progestin-induced mammary epithelial proliferation and carcinogenesis. *Nature* 468(7320), 103–107 (2010).

37 Kariagina A, Aupperlee MD, Haslam SZ. Progesterone receptor isoform functions in normal breast development and breast cancer. *Crit. Rev. Eukaryot. Gene Expr.* 18(1), 11–33 (2008).

38 Brisken C, O'Malley B. Hormone action in the mammary gland. *Cold Spring Harb. Perspect. Biol.* 2(12), a003178 (2010).

39 Clarke RB, Howell A, Potten CS, Anderson E. Dissociation between steroid receptor expression and cell proliferation in the human breast. *Cancer Res.* 57(22), 4987–4991 (1997).

40 Russo J, Ao X, Grill C, Russo IH. Pattern of distribution of cells positive for estrogen receptor α and progesterone receptor in relation to proliferating cells in the mammary gland. *Breast Cancer Res. Treat.* 53(3), 217–227 (1999).

41 Seagroves TN, Lydon JP, Hovey RC, Vonderhaar BK, Rosen JM. C/EBPβ (CCAAT/enhancer binding protein) controls cell fate determination during mammary gland development. *Mol. Endocrinol.* 14(3), 359–368 (2000).

42 Brisken C, Park S, Vass T *et al.* A paracrine role for the epithelial progesterone receptor in mammary gland development. *Proc. Natl Acad. Sci. USA* 95(9), 5076–5081 (1998).

43 Beleut M, Rajaram RD, Caikovski M *et al.* Two distinct mechanisms underlie progesterone-induced proliferation in the mammary gland. *Proc. Natl Acad. Sci. USA* 107(7), 2989–2994 (2010).

- **Delineates separate mechanism for progestin induced proliferation of PR-positive (cell-autonomous) and PR-negative (paracrine-stimulated) mammary epithelial cells.**

44 Brisken C, Heineman A, Chavarria T *et al.* Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling. *Genes Dev.* 14(6), 650–654 (2000).

45 Kariagina A, Aupperlee MD, Haslam SZ. Progesterone receptor isoforms and proliferation in the rat mammary gland during development. *Endocrinology* 148(6), 2723–2736 (2007).

- **Characterizes PR-A and -B in rat mammary glands and demonstrates that PR-B mediates proliferation in this model.**

46 Arpino G, Weiss H, Lee AV *et al.* Estrogen receptor-positive, progesterone receptor-negative breast cancer: association with growth factor receptor expression and tamoxifen resistance. *J. Natl Cancer Inst.* 97(17), 1254–1261 (2005).

47 Moore MR, Conover JL, Franks KM. Progestin effects on long-term growth, death, and Bcl-xL in breast cancer cells. *Biochem. Biophys. Res. Commun.* 277(3), 650–654 (2000).

48 Lydon JP, Ge G, Kittrell FS, Medina D, O'Malley BW. Murine mammary gland carcinogenesis is critically dependent on progesterone receptor function. *Cancer Res.* 59(17), 4276–4284 (1999).

49 Poole AJ, Li Y, Kim Y *et al.* Prevention of Brca1-mediated mammary tumorigenesis in mice by a progesterone antagonist. *Science* 314(5804), 1467–1470 (2006).

50 Lanari C, Lamb CA, Fabris VT *et al.* The MPA mouse breast cancer model: evidence for a role of progesterone receptors in breast cancer. *Endocr. Relat. Cancer* 16(2), 333–350 (2009).

51 Wiehle RD, Lantvit DD, Yamada T, Christov K. CDB-4124, a progesterone receptor modulator, inhibits mammary carcinogenesis by supressing cell proliferation and inducing apoptosis. *Cancer Prev. Res. (Phila.)* 4(3), 414–424 (2011).

52 Perrault D, Eisenhauer EA, Pritchard KI *et al.* Phase II study of the progesterone antagonist mifepristone in patients with untreated metastatic breast carcinoma: a National Cancer Institute of Canada Clinical Trials Group study. *J. Clin. Oncol.* 14(10), 2709–2712 (1996).

53 Robertson JF, Willsher PC, Winterbottom L, Blamey RW, Thorpe S. Onapristone, a progesterone receptor antagonist, as first-line therapy in primary breast cancer. *Eur. J. Cancer* 35(2), 214–218 (1999).

54 Chlebowski RT, Anderson GL, Gass M *et al.* Estrogen plus progestin and breast cancer incidence and mortality in postmenopausal women. *JAMA* 304(15), 1684–1692 (2010).

- •• **Large-scale clinical trial linking the progesterone component of hormone replacement therapy to increased risk of invasive breast cancer.**

55 Beral V. Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet* 362(9382), 419–427 (2003).

56 Hagan CR, Regan TM, Dressing GE, Lange CA. Ck2-dependent phosphorylation of progesterone receptors (PR) on Ser81 regulates PR-B-isoform-specific target gene regulation in breast cancer cells. *Mol. Cell Biol.* (In Press) (2011).

57 Bernstein L. Epidemiology of endocrine-related risk factors for breast cancer. *J. Mammary Gland Biol. Neoplasia* 7(1), 3–15 (2002).

58 Brisken C, Duss S. Stem cells and the stem cell niche in the breast: an integrated hormonal and developmental perspective. *Stem Cell Rev.* 3(2), 147–156 (2007).

59 Harmes DC, DiRenzo J. Cellular quiescence in mammary stem cells and breast tumor stem cells: got testable hypotheses? *J. Mammary Gland Biol. Neoplasia* 14(1), 19–27 (2009).

60 Shackleton M, Vaillant F, Simpson KJ *et al.* Generation of a functional mammary gland from a single stem cell. *Nature* 439(7072), 84–88 (2006).

61 Smith GH Label-retaining epithelial cells in mouse mammary gland divide asymmetrically and retain their template DNA strands. *Development* 132(4), 681–687 (2005).

62 Booth BW, Boulanger CA, Smith GH. Selective segregation of DNA strands persists in long-label-retaining mammary cells during pregnancy. *Breast Cancer Res.* 10(5), R90 (2008).

63 Booth BW, Smith GH. Estrogen receptor-α and progesterone receptor are expressed in label-retaining mammary epithelial cells that divide asymmetrically and retain their template DNA strands. *Breast Cancer Res.* 8(4), R49 (2006).

64 Asselin-Labat ML, Shackleton M, Stingl J *et al.* Steroid hormone receptor status of mouse mammary stem cells. *J. Natl Cancer Inst.* 98(14), 1011–1014 (2006).

65 Clarke RB. Isolation and characterization of human mammary stem cells. *Cell Prolif.* 38(6), 375–386 (2005).

66 Dontu G, Al-Hajj M, Abdallah WM, Clarke MF, Wicha MS. Stem cells in normal breast development and breast cancer. *Cell Prolif.* 36(Suppl. 1) 59–72 (2003).

67 Schramek D, Leibbrandt A, Sigl V *et al.* Osteoclast differentiation factor RANKL controls development of progestin-driven mammary cancer. *Nature* 468(7320), 98–102 (2010).

68 Tanos T, Brisken C. High hopes for RANKL: will the mouse model live up to its promise? *Breast Cancer Res.* 13(1), 302 (2011).

69 Ayyanan A, Civenni G, Ciarloni L *et al.* Increased Wnt signaling triggers oncogenic conversion of human breast epithelial cells by a Notch-dependent mechanism. *Proc. Natl Acad. Sci. USA* 103(10), 3799–3804 (2006).

70 Dontu G, Jackson KW, McNicholas E *et al.* Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells. *Breast Cancer Res.* 6(6), R605–R615 (2004).

71 Horwitz KB, Dye WW, Harrell JC, Kabos P, Sartorius CA. Rare steroid receptor-negative basal-like tumorigenic cells in luminal subtype human breast cancer xenografts. *Proc. Natl Acad. Sci. USA* 105(15), 5774–5779 (2008).

- **Describes a rare population of tumor-initiating cells in T47D breast cancer cell xenografts that are enriched upon progestin administration.**

72 Horwitz KB, Sartorius CA. Progestins in hormone replacement therapies reactivate cancer stem cells in women with preexisting breast cancers: a hypothesis. *J. Clin. Endocrinol. Metab.* 93(9), 3295–3298 (2008).

73 Groshong SD, Owen GI, Grimison B *et al.* Biphasic regulation of breast cancer cell growth by progesterone: role of the cyclin-dependent kinase inhibitors, p21 and p27(Kip1). *Mol. Endocrinol.* 11(11), 1593–1607 (1997).

74 Allred DC, Medina D. The relevance of mouse models to understanding the development and progression of human breast cancer. *J. Mammary Gland Biol. Neoplasia* 13(3), 279–288 (2008).

75 Lange CA, Yee D. Progesterone and breast cancer. *Womens Health (Lond. Engl.)* 4(2), 151–162 (2008).

76 Benakanakere I, Besch-Williford C, Ellersieck MR, Hyder SM. Regression of progestin-accelerated 7,

12-dimethylbenz[a]anthracene-induced mammary tumors in Sprague–Dawley rats by p53 reactivation and induction of massive apoptosis: a pilot study. *Endocr. Relat. Cancer* 16(1), 85–98 (2009).

77 Cerliani JP, Giulianelli S, Sahores A *et al.* Mifepristone inhibits MPA-and FGF2-induced mammary tumor growth but not FGF2-induced mammary hyperplasia. *Medicina (B. Aires)* 70(6), 529–532 (2010).

78 Graham JD, Yeates C, Balleine RL *et al.* Characterization of progesterone receptor A and B expression in human breast cancer. *Cancer Res.* 55(21), 5063–5068 (1995).

79 Anderson E. The role of oestrogen and progesterone receptors in human mammary development and tumorigenesis. *Breast Cancer Res.* 4(5), 197–201 (2002).

80 Clarke CA, Glaser SL, Uratsu CS *et al.* Recent declines in hormone therapy utilization and breast cancer incidence: clinical and population-based evidence. *J. Clin. Oncol.* 24(33), e49–e50 (2006).

81 Hofseth LJ, Raafat AM, Osuch JR *et al.* Hormone replacement therapy with estrogen or estrogen plus medroxyprogesterone acetate is associated with increased epithelial proliferation in the normal postmenopausal breast. *J. Clin. Endocrinol. Metab.* 84(12), 4559–4565 (1999).

82 Lee S, Kolonel L, Wilkens L *et al.* Postmenopausal hormone therapy and breast cancer risk: the Multiethnic Cohort. *Int. J. Cancer* 118(5), 1285–1291 (2006).

83 Ross RK, Paganini-Hill A, Wan PC, Pike MC. Effect of hormone replacement therapy on breast cancer risk: estrogen versus estrogen plus progestin. *J. Natl Cancer Inst.* 92(4), 328–332 (2000).

84 Rossouw JE, Anderson GL, Prentice RL *et al.* Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. *JAMA* 288(3), 321–333 (2002).

85 Shen T, Horwitz KB, Lange CA. Transcriptional hyperactivity of human progesterone receptors is coupled to their ligand-dependent down-regulation by mitogen-activated protein kinase-dependent phosphorylation of serine 294. *Mol. Cell Biol.* 21(18), 6122–6131 (2001).

86 Hennighausen L, Robinson GW. Signaling pathways in mammary gland development. *Dev. Cell* 1(4), 467–475 (2001).

87 Fan P, Yue W, Wang JP *et al.* Mechanisms of resistance to structurally diverse antiestrogens differ under premenopausal and postmenopausal conditions: evidence from *in vitro* breast cancer cell models. *Endocrinology* 150(5), 2036–2045 (2009).

88 Massarweh S, Osborne CK, Creighton CJ *et al.* Tamoxifen resistance in breast tumors is driven by growth factor receptor signaling with repression of classic estrogen receptor genomic function. *Cancer Res.* 68(3), 826–833 (2008).

89 Ring A, Dowsett M. Mechanisms of tamoxifen resistance. *Endocr. Relat. Cancer* 11(4), 643–658 (2004).

90 Mote PA, Bartow S, Tran N, Clarke CL. Loss of co-ordinate expression of progesterone receptors A and B is an early event in breast carcinogenesis. *Breast Cancer Res. Treat.* 72(2), 163–172 (2002).

91 Cui X, Schiff R, Arpino G, Osborne CK, Lee AV. Biology of progesterone receptor loss in breast cancer and its implications for endocrine therapy. *J. Clin. Oncol.* 23(30), 7721–7735 (2005).

92 Cui X, Zhang P, Deng W *et al.* Insulin-like growth factor-I inhibits progesterone receptor expression in breast cancer cells via the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway: progesterone receptor as a potential indicator of growth factor activity in breast cancer. *Mol. Endocrinol.* 17(4), 575–588 (2003).

93 Chu I, Arnaout A, Loiseau S *et al.* Src promotes estrogen-dependent estrogen receptor α proteolysis in human breast cancer. *J. Clin. Invest.* 117(8), 2205–2215 (2007).

94 Daniel AR, Knutson TP, Lange CA. Signaling inputs to progesterone receptor gene regulation and promoter selectivity. *Mol. Cell Endocrinol.* 308(1–2), 47–52 (2009).

95 Levin ER. Bidirectional signaling between the estrogen receptor and the epidermal growth factor receptor. *Mol. Endocrinol.* 17(3), 309–317 (2003).

CURRICULUM VITAE

Name **Christy R. Hagan**

Address University of Minnesota
Department of Medicine & Women's Cancer Program
MMC 806
420 Delaware St SE
Minneapolis, MN 55455
(612) 624-1971
hagan018@umn.edu

EDUCATION

2000-2006 University of Chicago, Chicago, IL
Committee on Cancer Biology
Mentor: Charles Rudin, MD, PhD
Degree granted: Ph.D. in Cancer Biology
Dissertation: *Induction of mobile genetic elements following exposure to DNA-damaging agents*

1994-1998 Colorado College, Colorado Springs, CO
Degree granted: B.A. in Biochemistry, *cum laude*

RESEARCH EXPERIENCE

2008 to current Postdoctoral Fellowship, University of Minnesota
Mentor: Carol Lange, PhD
Research Focus: Studying the regulation of the progesterone receptor by MAPKs.

2006 to 2008 Postdoctoral Fellowship, Northwestern University
Mentor: Vincent Cryns, MD
Research Focus: Studying role of αB-crystallin in protecting breast cancer cells from chemotherapy-induced apoptosis.

2000 to 2006 Graduate Student, University of Chicago, Chicago, IL
Mentor: Charles Rudin, MD, PhD
Research Focus: Defining pathways responsible for activating movement of retrotransposons in response to genotoxic stress. Research also entails investigating how retrotransposition of these elements may be related to the etiology of secondary malignancies.

1998-2000 Pre-Doctoral Fellow, Human Gene Therapy Research Institute,
Des Moines, IA
Mentor: Charles Link, MD, PhD

Research Focus: Concentrating radioisotope in cancer cells using the sodium/iodide symporter gene as a novel approach to cancer gene therapy. Secondary project involved restoring DNA repair activity in xeroderma pigmentosum cells using the cloned T4 endonuclease V gene.

1997-1998 Organic Chemistry Lab Assistant, Colorado College,
Colorado Springs, CO
Primary Duties: Instructed students in basic organic chemistry lab techniques, such as NMR, IR, TLC and MS; responsible for student safety in the lab; graded lab related assignments, including lab notebooks, tests, and lab technical competency tests.

HONORS, AWARDS AND PROFESSIONAL MEMBERSHIPS

Honors and Awards

Brigid G. Leventhal Women in Cancer Research Scholar, awarded for the presentation of a meritorious scientific paper, 96th Annual Meeting of the American Association for Cancer Research, 2005

University of Chicago Doolittle Fellowship, awarded to students with outstanding academic achievement for travel to a scientific meeting, 2005

University of Chicago Women's Board Travel Fellowship, awarded to students presenting a meritorious abstract at a scientific meeting, 2005

University of Chicago Doolittle Fellowship, awarded to students with outstanding academic achievement for travel to a scientific meeting, 2003

Aventis Scholar in Training, awarded to students and fellows whose proffered papers are highly rated by the judging committee, 93rd Annual Meeting of the American Association for Cancer Research, 2002

Cum laude, B.A. in Biochemistry, Colorado College, 1998

Alpha Lambda Delta Honor Society, Colorado College, 1998

Outstanding Achievement in Biochemistry Award, awarded to the graduating student with the strongest academic record, Colorado College, 1998

Dean's List, Colorado College, 1994-1998

Grants/Funding

DOD Breast Cancer Research Program Post-doctoral Award; 2009-2012

Komen Grants Program, Post-doctoral Fellowship Award; awarded 2009-2012, declined due to acceptance of DOD award

American Cancer Society, Post-doctoral Fellowship; awarded for 2009-2011, declined due to acceptance of DOD award

Komen Grants Program, Post-doctoral Fellowship Award; awarded 2008, declined due to institutional relocation

Institutional National Research Service Award (University of Minnesota), National Cancer Institute, awarded for postdoctoral fellowship salary support, 2008-2009

Institutional National Research Service Award (Northwestern University), National Cancer Institute, awarded for postdoctoral fellowship salary support, 2007-2008

Penny Severns Post-doctoral Fellowship, Illinois Department of Public Health; awarded for 2007 fiscal year

Institutional National Research Service Award (Northwestern University), National Cancer Institute, awarded for postdoctoral fellowship salary support, 2006-2007

Professional Memberships

Member, Women in Endocrinology, 2008-present

Member, The Endocrine Society, 2008-present

Member, Women in Cancer Research, 2003-present

Associate Member, American Association for Cancer Research, 2002-present

Member, American Association for the Advancement of Science, 1997-present

BIBLIOGRAPHY

Publications

Hagan, C.R., Regan, T.M., Dressing, G.E. and Lange, C.A. ck2-Dependent Phosphorylation of Progesterone Receptors (PR) on Ser81 Regulates PR-B-Isoform-Specific Target Gene Expression in Breast Cancer Cells. ***Mol Cell Biol*** 2011 Jun; 31(12): 2439-2452.

Hagan, C.R., Faivre, E.J., and Lange, C.A. Scaffolding Actions of Membrane-Associated Progesterone Receptors. ***Steroids*** 2009 Jul;74(7):568-72.

Dressing GE, **Hagan CR**, Knutson TP, Daniel AR, Lange CA. Progesterone receptors act as sensors for mitogenic protein kinases in breast cancer models. ***Endocrine-Related Cancer*** 2009 Jun;16(2):351-61.

Carbajal L, Deng J, Dressing GE, **Hagan CR**, Lange CA, Hammes SR. Meeting review: Extra-nuclear steroid receptors-Integration with multiple signaling pathways. ***Steroids*** 2009 Jul;74(7):551-4.

Hagan, C.R. and Rudin, C.M. DNA cleavage and Trp53 differentially effect SINE transcription. ***Genes Chromosomes and Cancer*** 2007 Mar;46(3):248-60.

Hagan, C.R., Sheffield R.F., and Rudin, C.M. Human Alu element retrotransposition induced by genotoxic stress. ***Nature Genetics*** 2003 Nov;35(3): 219-20.

Heltemes, L.M., **Hagan, C.R.**, Mitrofanova, E.E., Panchal, R.G., Guo, J. and Link, C.J. The sodium iodide symporter gene permits more effective radioisotope concentration than the human sodium iodide symporter gene in human and rodent cells. ***Cancer Gene Therapy*** 2003 10(1): 14-22.

Mitrofanova E.**, Hagan C.**, Qi J., Seregina T., Link C. Jr. Sodium iodide symporter/radioactive iodine system has more efficient antitumor effect in three-dimensional spheroids. ***Anticancer Research*** 2003 May-Jun;23(3B):2397-404.

Hagan, C.R., and Rudin, C.M. Mobile Genetic Element Activation and Genotoxic Cancer Therapy: Potential Clinical Implications. ***American Journal of PharmacoGenomics*** 2002 2(1): 25-35.

Brickley, D.R., Mikosz, C.A., **Hagan, C.R.,** and Conzen, S.D. Regulation of serum and glucocorticoid-induced protein kinase (SGK-1) by ubiquitination. ***Journal of Biological Chemistry*** 2002 277(45): 43064-70.

Invited Presentations

Hagan, C.R., Hillard, C.J., Lange, C.A. Signaling Inputs to Progesterone Receptor Action in Breast Cancer Models. FASEB Summer Research Conference: The Physiology of Integrated Nuclear and Extranuclear Steroid Signaling. August 8-13, 2010.

Hagan, C.R., Hillard, C.J., Lange, C.A. A common docking domain in the progesterone receptor mediates an interaction with MAPK-phosphatase 3. University of Minnesota Masonic Cancer Center Symposium. June 10, 2010.

Abstracts

Hagan, C.R., Regan, T.M., Dressing, G.E. and Lange, C.A. ck2-Dependent Phosphorylation of Progesterone Receptors (PR) on Ser81 Regulates PR-B-Isoform-Specific Target Gene Expression in Breast Cancer Cells. 102nd Annual Meeting of the American Association for Cancer Research. April 2-6, 2011.

Hagan, C.R., Hillard, C.J., Faivre, E.J., Lange, C.A. A common docking domain in the progesterone receptor mediates an interaction with MAPK-phosphatase 3. Jensen Symposium on Nuclear Receptors. October 14-16, 2009.

Hagan, C.R., Hillard, C.J., Faivre, E.J., Lange, C.A. A common docking domain in the progesterone receptor mediates an interaction with MAPK-phosphatase 3. Gordon Research Conference: Hormone Action in Development and Cancer. July 26-31, 2009.

Hagan, C.R., Hillard, C.J., Lange, C.A. Exploring the role of PR/MEK complex formation in Breast Cancer Models. FASEB Summer Research Conference: Extra-Nuclear Steroid Receptors: Integration with Multiple Signaling Pathways. July 27- August 1, 2008.

Hagan, C.R., Rudin, C.M. JNK is required for SINE transcriptional response following DNA damage. 97th Annual Meeting of the American Association for Cancer Research. April 1-5, 2006.

Hagan, C.R., Rudin, C.M. DNA-damage activates transcription of Short Interspersed Elements. FASEB Summer Research Conference: Mammalian Mobile Elements. June 4-9, 2005.

Hagan, C.R., Rudin, C.M. DNA-damage activates transcription of Short Interspersed Elements. 96th Annual Meeting of the American Association for Cancer Research. April 16 -20, 2005. Anaheim, CA.

Hagan, C.R., Rudin, C.M. Transcriptional activation of Short Interspersed Elements by genotoxic exposure is attenuated by functional p53. 94th Annual Meeting of the American Association for Cancer Research. July 11-14, 2003. Washington, D.C.

Hagan, C.R., Sheffield, R.F., Rudin, C.M. Induction of Genomic Mobility of SINE Retrotransposable Elements by Genotoxic Exposure. 93rd Annual Meeting of the American Association for Cancer Research, April 6-10, 2002, San Francisco, CA.

Hagan, C.R., Heltemes, L.M., Panchal R.G., Guo, J., Link, C.J. Iodine Uptake and Cell Death in Various Cancer Cell Lines Using the Rat and Human Sodium Iodide Symporter Gene. *Molecular Therapy* 2000 May; 1 (5): S165.

Mitrofanova, E.E., **Hagan, C.R.**, Link, C.J. The Effect of ^{131}I on the Growth of Muticellular Tumor Spheroids Expressing the Sodium Iodide Symporter. *Molecular Therapy* 2000 May; 1 (5): S165.

Guo, J., **Hagan, C.R.**, Panchal, R.G., Mitrofanova, E.E., Qi, J., Wang, S., Link, C.J. Efficient Uptake of Radioisotope into Human Prostate Adenocarcinoma after Rat Sodium Iodide Symporter Gene Transfer Using HSV-1 Amplicon Vector. *Molecular Therapy* 2000 May; 1 (5): S237.

Link, C.J., Heltemes, L.M., Panchal, R.G., Guo, J., **Hagan, C.R.** Radioisotope concentrator gene therapy for cancer with the sodium/iodide symporter gene. American Society of Gene Therapy, Second Annual Meeting, Washington, D.C., June 9-12, 1999.

Link, C.J., Panchal, R.G., **Hagan, C.R.** Heltemes, L.M. Gene therapy for cancer with the sodium/iodide symporter gene. Sixth International Conference of Anticancer Research, October 21-25, 1998, Halkidiki, Greece.